REMARKS

Claims 1-3, 5-7 and 9-11 are all the claims pending in the application.

Claim Rejections under 35 U.S.C. § 103

1. Claims 1-3, 5, 6 and 9-11 remain rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Goodwin *et al.* (U.S. Patent No. 5,496,722; "Goodwin #1"), Baker *et al.* (In Vitro. Cell Dev. Biol., vol 33, page 358, 1997; "Goodwin #2"), Goodwin *et al.* (In Vitro. Cell Dev. Biol., vol 33, page 366, 1997; "Goodwin #3") and Schwarz *et al.* (USP 5,026,650; "Schwarz") in view of Unsworth *et al.* (Nature Medicine; "Unsworth"), Wikipedia, Bock *et al.* (Tissue Engineering of Cartilage and Bone; "Bock") and Bartlett (Ovarian Cancer Methods and Protocols).

Applicants previously asserted that the combination of the cited art fails to teach or suggest the confluent two-dimensional (2D) culture of mesenchymal cells prior to culture in RWV. Pages 4-5, *Amendment filed July 28, 2008*. In response, the Examiner asserts that culturing 2D cells to confluence is a known technique in the art and that applying this known technique to the method of Goodwin #1 is obvious in the absence of evidence to the contrary or unexpected results.

Applicants respectfully disagree and traverse the rejection as follows.

First, regarding Applicants' previous argument that one of ordinary skill in the art would have no reasonable expectation of success in using a confluent 2D culture prior to differentiation for mesenchymal stem cells, the Examiner disagrees and cites another reference, Current Protocols in Cell Biology. The Examiner states that this reference teaches that "[c]ultures should be 75% to 100% confluent when selected for subculture" and that it would be obvious to one of

ordinary skill in the art to culture cells to confluence when using traditional 2D culture techniques.

In response, Applicants respectfully point out that, the cited Current Protocols reference refers to cells only in generic terms and is silent regarding any culture method specifically for mesenchymal stem cells. In fact, it was well-known in the art at the time of the invention that the culture of mesenchymal stem cells is exceptional in that mesenchymal stem cells are not cultured by the general confluent culture method. Rather, mesenchymal stem cells are passaged prior to reaching such confluency. Further, this is done to avoid the undesirable result of uncontrolled differentiation that occurs when the cells are cultured to confluency.

In fact, at least by 1998, non-confluent culture for mesenchymal stem cells was the common practice in the art as shown in Majumdar *et al.* (Submitted hereinwith; Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells, *Journal of Cellular Physiology*, Column 17, Issue 1, Pages 57-66). Moreover, Metzger (Submitted hereinwith; Cardiac cell and gene transfer: principles, protocols, and applications, *Humana Press*, 2003, page 77) teaches a non-confluent culturing protocol for mesenchymal stem cells that specifies only 80% confluency for the mesenchymal stem cell culture. Furthermore, Alhadlaq *et al.*, (Submitted hereinwith; Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells, *J Dent Res,* 82(12): 951-956, 2003) applies the non-confluent culturing protocol for mesenchymal stem cells prior to 3D differentiation. Indeed, culturing mesenchymal stem cells to confluence in 2D prior to 3D differentiation was not the accepted practice and was not an obvious technique in the art at the time of invention.

Thus, one of ordinary skill in the art would have no reasonable expectation of success in differentiating mesenchymal stem cells to form cartilage tissue by using a confluent 2D culture prior to differentiation. As M.P.E.P. § 2143.02 states, "[r]easonable [e]xpectation of [s]uccess [i]s [r]equired. ... [o]bviousness does not require absolute predictability, however, at least some degree of predictability is required. Evidence showing there was no reasonable expectation of success may support a conclusion of nonobviousness." See *In re Rinehart*, 531 F.2d 1048, 189 USPQ 143 (CCPA 1976); See also *Amgen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1207-08, 18 USPQ2d 1016, 1022-23 (Fed. Cir.), *cert. denied*, 502 U.S. 856 (1991); *In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988).

Second, as explained at page 6 of Amendment filed July 28, 2008, Applicants respectfully point out that Applicants are not claiming an expansion or production of mesenchymal cells in any of the pending claims. Rather, the presently claimed invention aims to promote differentiation of mesenchymal stem cells to form tissues. Specifically, the presently claimed invention uses a confluent 2D culture of mesenchymal stem cells to promote the cells' extracellular matrix production to simulate a natural cartilage tissue environment. The extracellular matrix produced during the confluent 2D culture supports the mesenchymal stem cells to differentiate and produce more extracellular matrix in the later 3D culture. Such an advantageous effect of the confluent 2D culture for mesenchymal stem cell prior to a 3D culture was not obvious to one of ordinary in the art at the time of invention.

As discussed at page 6 of Amendment filed July 28, 2008, Applicants assert that the Office Action fails to provide a reason as to why one of ordinary skill in the art would pursue the

techniques used to expand chondrocytes or ovarian tumor cells in the differentiation of mesenchymal cells.

In maintaining the rejection, the Examiner contends that Goodwin #1 discloses promoting differentiation of mesenchymal stem cells to form cartilage tissue by producing cartilage tissue expressing Type II collagen from bone marrow mesenchymal cells. Page 4, *Office Action of November 24, 2008*. However, Applicants respectfully point out that, since confluent culture technique for mesenchymal stem cells prior to their differentiation is not suggested by Goodwin #1, the Office still fails to set forth a reason why one of ordinary skill in the art would modify Goodwin's mesenchymal stem cell culture technique to incorporate the confluent culture techniques to expand chondrocytes or ovarian tumor cells as recited in the present claims.

As the Supreme Court recently discussed, the "apparent reason to combine the known elements in a fashion claimed by the [claims] at issue ... should be made explicit." *KSR Int'l Co. v. Teleflex, Inc.* No 04-1350 slip op. at 14 (U.S. Apr. 30, 2007). Rather than indicating why one of skill in the art would choose to combine the references in a method for cell differentiation, the Examiner is silent.

Third, in maintaining the rejection, the Examiner states that Applicants have argued that the Examiner has not recognized the order of the steps of the method. Page 2, *Office Action of November 24, 2008*. However, Applicants respectfully submit that Applicants have not made such an argument. The Examiner appears to have misunderstood Applicants' previous argument that the subculture from the confluent 2D cell culture to three-dimensional (3D) culture is a single element. Pages 4-5, *Amendment filed July 28, 2008*.

In making the rejection, the Examiner also asserts that Goodwin #2 and #3 disclose 2D culture prior to 3D culture, and Bock and Bartlett disclose confluent 2D culture prior to passage. Page 7, *Office Action of November 24, 2008* and page 6, *Office Action of April 28, 2008*. The Examiner appears to take a single element, the subculture from confluent 2D culture to 3D culture, and separate it into two elements, namely the confluent 2D culture and the order of the 2D and 3D cultures. However, as set forth in the previous Amendment filed July 28, 2008, Applicants respectfully submit that subculturing mesenchymal stem cells from confluent two-dimensional culture to three-dimensional culture is a single element, and the combination of the cited references fails to teach or suggest this element.

To establish *prima facie* obviousness of a claimed invention, all the cited references must recite all the claim limitations. *In re Royka,* 490 F.2d 981, 984 (CCPA 1974). For the reasons previously presented above, Applicants assert that the combination of cited references fails to teach or suggest all the claim limitations of the invention, either explicitly or inherently. In particular, these references do not expressly or inherently teach "subculturing the cells from confluent two-dimensional culture to three-dimensional culture." Thus, these references do not support a *prima facie* case of obviousness.

Fourth, Applicants submit that the claimed invention discloses an unexpectedly superior result of not requiring a carrier for the 3D mesenchymal stem cell culture. Specifically, all 3D cultures disclosed by Goodwin #1, #2, and #3 require the use of Cytodex-3 microcarrier. *See* Columns 12, last paragraph, of Goodwin #1 and Materials and Method section of Goodwin #2 and #3. On the other hand, the present invention does not require such a carrier in the 3D culture to form a desired tissue. *See* Example 1.

As M.P.E.P. § 716.02(a) states, the "[p]resence of a property not possessed by the prior art is evidence of nonobviousness. *In re Papesch*, 315 F.2d 381, 137 USPQ 43 (CCPA 1963)." Thus, Applicants respectfully submit that the presently claimed invention is not obvious over the cited references at least because of its unexpectedly superior result of not requiring a carrier for the 3D mesenchymal stem cell culture.

For the reasons set forth above, Applicants respectfully request that the above rejection under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

2. Claims 1-3, 5-7 and 9-11 remain rejected under 35 U.S.C. § 103(a) as being unpatentable over Goodwin #1, #2, #3 and Schwarz, and further in view of Yan *et al.* (US 2002/0168763; "Yan") and Simpson *et al.* (US 2002/0090725; "Simpson"). The Examiner relies on Goodwin #1, #2, #3 and Schwarz to reject Claims 1-3, 5, 6 and 9-11 as described above and further rejects Claim 7 in view of Yan and Schwarz.

Claim 7 directly depends on claim 1. Therefore, the reasons presented above for claim 1 also apply in the present rejection. Namely, it has been shown above not only that one of ordinary skill in the art would have no reasonable expectation of success in combining references, but also that there is no reason for one of skill in the art to make the proposed modification. Moreover, the combination of Goodwin #1, #2, #3 and Schwarz fails to teach or suggest subculture from confluent 2D culture to 3D culture. Neither Yan nor Simpson cures this deficiency. Thus, these references do not support a *prima facie* case of obviousness.

Additionally, Applicants respectfully assert that dependent Claim 7 is also allowable because the combination of the cited references fails to teach or suggest making cartilage tissue by adding dexamethasone as recited in Claim 7.

At Page 11 of the Office Action, the Examiner contends that the use of dexamethasone (DEX) in culture medium to grow bone marrow cells is obvious in view of Yan. However, while Yan adds DEX in medium to differentiate pluripotent HS stem cells into a hepatic cell line, DEX is absent in the medium to promote the mesodermal differentiation into bone marrow and cartilage. *See* Yan at [307], [310], and [330]. The rest of the cited references fail to cure this deficiency of Yan.

Applicants therefore respectfully request that the above rejection under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

CONCLUSION

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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Date: April 24, 2009

Phenotypic and Functional Comparison of Cultures of Marrow-Derived Mesenchymal Stem Cells (MSCs) and Stromal Cells

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Mesenchymal stem cells (MSCs) are a population of pluripotent cells within the bone marrow microenvironment defined by their ability to differentiate into cells of the osteogenic, chondrogenic, tendonogenic, adipogenic, and myogenic lineages. We have developed methodologies to isolate and culture-expand MSCs from human bone marrow, and in this study, we examined the MSC's role as a stromal cell precursor capable of supporting hematopoietic differentiation in vitro. We examined the morphology, phenotype, and in vitro function of cultures of MSCs and traditional marrow-derived stromal cells (MDSCs) from the same marrow sample. MSCs are morphologically distinct from MDSC cultures, and flow cytometric analyses show that MSCs are a homogeneous cell population devoid of hematopoietic cells. RT-PCR analysis of cytokine and growth factor mRNA in MSCs and MDSCs revealed a very similar pattern of mRNAs including IL-6, -7, -8, -11, -12, -14, and -15, M-CSF, Flt-3 ligand, and SCF. Steady-state levels of IL-11 and IL-12 mRNA were found to be greater in MSCs. Addition of IL-1α induced steady-state levels of G-CSF and GM-CSF mRNA in both cell preparations. In contrast, IL-1α induced IL-1α and LIF mRNA levels only in MSCs, further emphasizing phenotypic differences between MSCs and MDSCs. In long-term bone marrow culture (LTBMC), MSCs maintained the hematopoietic differentiation of $CD34^+$ hematopoietic progenitor cells. Together, these data suggest that MSCs represent an important cellular component of the bone marrow microenvironment. J. Cell. Physiol. 176:57–66, 1998. © 1998 Wiley-Liss, Inc.

The bone marrow is comprised of hematopoietic cells and adherent stromal cells of nonhematopoietic origin which together with the extracellular matrix provide a supportive scaffolding termed the bone marrow microenvironment. The cellular components of the marrow microenvironment include reticular endothelial cells, macrophages, adipocytes, fibroblasts, and osteogenic precursor cells (Beresfords, 1989; Dorshkind, 1990). By promoting cell-to-cell interactions (Williams, 1994), the expression and presentation of cytokines and growth factors, and the secretion of extracellular matrix proteins, the marrow microenvironment provides a favorable platform for the localization, self-renewal, and differentiation of hematopoietic stem cells (HSCs).

Histological analysis of the hematopoietic bone marrow indicates the presence of a complex array of niches where specific cell types play distinct functions in the regulation of hematopoiesis (Weiss, 1995). Although numerous studies with mixed stromal cell cultures have advanced our understanding of the role of the bone marrow microenvironment in hematopoiesis, detailed molecular and functional characterization of the individual cellular components of the stroma is incomplete. Immortalized bone marrow stromal cells (Dorshkind and Landreth, 1992; Deryugina and Muller-Sieburg, 1993; Roecklein and Torok-Storb, 1995; Wineman et al., 1993; Mosca et al., 1995) have been used in long-term bone marrow cultures (LTBMC) to further define the heterogeneity in the marrow microenvironment (Dexter et al., 1977). The major disadvantages of relying on transformed and immortalized cell lines to determine the functional elements of the marrow microenvironment lies in the potential of these cells to undergo morphologic, phenotypic, and regulatory changes that make them unpredictable surrogates for their normal cell counterparts.

In addition to hematopoietic stem cells, the bone mar-

Abbreviations: BMM bone marrow microenvironment; HSC hematopoietic stem cell; LTBMC long-term bone marrow culture; LTC-IC long-term culture-initiating cell; MDSC marrow-derived stromal cells; MSC mesenchymal stem cell.

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row microenvironment is also home to pluripotent nonhematopoietic precursor cells that have the ability to form specific connective tissues such as bone, cartilage, and the marrow stroma (Beresfords, 1989; Dorshkind, 1990; Caplan, 1991). These pluripotent precursors, termed mesenchymal stem cells (MSCs), have been shown to differentiate into various cell lineages, including osteoblasts (Haynesworth et al., 1992a), chondrocytes (Johnstone et al., 1996), and adipocytes (Pittenger et. al., 1996) when placed in appropriate in vitro and in vivo environments. Previous studies have also shown that MSCs secrete a number of cytokines such as interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Haynesworth et al., 1996). In this study, we examined the expression of cell surface molecules, cytokines, and growth factors by MSCs and test their ability to maintain hematopoiesis in LTBMC with $CD34^+$ hematopoietic progenitors. The analysis of MSCs was compared to MDSCs prepared from the same marrow sample.

MATERIALS AND METHODS
Isolation and culture expansion of MSCs and MDSCs

Bone marrow samples were collected from healthy human donors at the Johns Hopkins University under an Institutional Review Board-approved protocol. MSCs were isolated and cultured according to modifications of a previously reported method (Haynesworth et al., 1992a). Briefly, 25 ml of heparinized bone marrow was mixed with an equal volume of phosphate-buffered saline (PBS) (Life Technologies, Gaithersburg, MD) and centrifuged at 900g for 10 min at room temperature. Washed cells were resuspended in PBS to a final density of 4×10^7 cells/ml, and a 5 ml aliquot was layered over a 1.073 g/ml Percoll solution (Pharmacia, Piscataway, NJ) and centrifuged at 900g for 30 min. Mononuclear cells collecting at the interface were recovered, resuspended in human MSC medium, and plated at a density of 3×10^7 cells per 185 cm^2 Nunclon Solo flask (Nunc Inc., Naperville, IL). Human MSC medium consisted of Dulbecco's Modified Eagles Medium-Low Glucose (DMEM-LG) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Biocell Laboratories, Rancho Dominquez, CA) and 1% antibiotic-antimycotic solution (Life Technologies). The FBS used in MSC medium was selected based on its ability to maximize recovery and culture expansion of MSCs from bone marrow (Lennon et al., 1996). MSC cultures were maintained at 37°C in 5% CO_2 in air, with medium changes after 48 h and every 3–4 days thereafter. When the cultures reached 90% of confluence, cells were recovered by the addition of a solution containing 0.25% trypsin-EDTA (Life Technologies) and replated at a density of 1×10^6 cells per 185 cm^2 flask as passage 1 cells.

MDSCs were isolated from the same bone marrow sample using the following procedure. Bone marrow was mixed with an equal volume of PBS containing 2% bovine serum albumin (BSA) (Life Technologies), 0.6% sodium citrate (Sigma, St. Louis, MO), and 1% penicillin-streptomycin (Life Technologies), and aliquots were layered over Ficoll-Paque (1.077 gm/ml) (Pharmacia) and centrifuged at 800g for 20 min. The mononuclear cells that collected at the interface were suspended in LTBMC medium and plated at a density of 60×10^6 cells per 185 cm^2 flask and incubated at 37°C in 5% CO_2 in air. LTBMC medium consisted of MyeloCult H5100 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 1 μM hydrocortisone (Sigma). MyeloCult H5100 is made up of 12.5% FBS, 12.5% horse serum, 0.2 mM i-inositol, 20 mM folic acid, 0.1 mM 2-Mercaptoethanol, 2 mM L-glutamine in Alpha MEM. Medium was changed every 7 days, and primary cultures were recovered by trypsinization when the cells reached 90% of confluence and replated at a density of 1×10^6 cells per 185 cm^2 flask as passage 1 cells.

Flow cytometry

Analysis of cell surface molecules was performed on passage 1 cultures of MSCs and MDSCs using flow cytometry and the following procedure. Media was removed from flasks, and cell layers were washed twice with PBS and detached from the flask by incubation with a solution of 0.25% trypsin-EDTA for 5 min at room temperature. Cells were recovered by centrifugation and washed in flow cytometry buffer consisting of 2% BSA and 0.1% sodium azide (Sigma) in PBS. Aliquots (2×10^5 cells) were incubated with conjugated monoclonal antibodies, either SH-2-PE (Haynesworth et al., 1992b) and SB-10-FITC (Bruder et al., 1997) (Osiris Therapeutics Inc., Baltimore, MD) or anti-CD14-PE and anti-CD45-Cy-chrome (PharMingen, San Diego, CA). All incubations with antibodies were performed for 20 min, after which cells were washed with flow cytometry buffer. Washed cells were pelleted and resuspended in flow cytometry buffer containing 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). Nonspecific fluorescence was determined using equal aliquots of the cell preparation that were incubated with anti-mouse monoclonal antibodies (PharMingen). Data for both the MSCs and the MDSCs cultures were collected under identical parameters and analyzed by collecting 10,000 events on a Becton Dickinson Vantage instrument (San Jose, CA) using CellQuest software.

Long-term bone marrow cultures (LTBMC)

To establish LTBMC, we removed primary cultures of MSCs and MDSCs from the tissue culture flask by trypsinization. Cells were suspended at a density of 2×10^6 cells/ml in LTBMC medium and exposed to 16 Gy γ irradiation from a ^{137}Cs source. The irradiated cells were then plated in triplicate at a density of $3–5 \times 10^6$ cells per well in a six-well culture plate, and cultures were incubated overnight.

$CD34^+$ cells were isolated from the Ficoll (1.077 gm/ml) fraction of fresh bone marrow using the Dynal CD34 progenitor cell selection system (Dynal Inc., Lake Success, NY) according to the manufacturer's protocol. Generally, the total number of $CD34^+$ cells recovered were 1–2% of the total mononuclear cell fraction. Aliquots (1×10^5) $CD34^+$ cells were layered onto each well of either MSC or MDSCs, and the cocultures were incubated at 33°C in 5% CO_2 in air in LTBMC medium. Half of the medium was replaced weekly with fresh medium for 5 weeks. Experimental controls consisted of LTBMC of $CD34^+$ cells in the absence of preformed adherent monolayer cells.

After 5 weeks of coculture, nonadherent cells from the LTBMC were collected from the medium by centrifugation, and the adherent cells from each well were recovered by trypsinization. Cells from each fraction were resuspended in 0.3 ml of LTBMC medium, and the suspension was added to 2.7 ml of methylcellulose medium, MethoCult 4435 (Stem Cell Technologies). Aliquots (1 ml) of the cell mixture were plated in duplicate in 35 mm Nunc dishes (Nunc Inc.) and incubated at 37°C in 5% CO_2 in air. After 2 weeks, colonies composed of >50 cells were scored, and the numbers from both fractions were combined and used for statistical analysis.

RNA preparation and analysis

Passage 1 MSCs were incubated for 24 h in either MSC medium, MSC medium containing 10 units/ml IL-1α (Boehringer Mannheim, Indianapolis, IN), or LTBMC medium. Passage 1 MDSCs were maintained in either LTBMC medium or in LTBMC medium in which 10 units/ml IL-1α was added for 24 h. Total RNA was extracted from cultures of MSCs and MDSCs by modification of the method of Chirgwin et al. (1979). Briefly, cells were lysed in a solution consisting of 4 M guanidinium isothiocynate (Sigma), 0.03 M sodium acetate (Sigma), and 0.4 g/ml of cesium chloride (Life Technologies). Lysates were layered over 3 ml of 5.7 M CsCl and centrifuged for 18 h at 155,000g in a Beckman (Palo Alto, CA) SW40 rotor. RNA was dissolved in diethyl pyrocarbonate-(DEPC) (Sigma) treated water and precipitated by the addition of 1/10 volume of 0.3 M sodium acetate and 2 volumes of absolute ethanol. RNA was recovered by centrifugation and dissolved in DEPC-treated water at a concentration of 0.5 mg/ml.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using total RNA as a template, a RNA PCR core kit (Perkin-Elmer Cetus, Norfolk, CT), and the oligonucleotide primers (Operon, Alameda, CA) in Table 1. The RT-PCR products were analyzed by electrophoresis on a 2% agarose gel containing 1 μg/ml ethidium bromide.

For analysis of gene expression, we utilized the PCR ELISA kit (Boehringer Mannheim) and the procedure recommended by the manufacturer. Briefly, PCR was performed in the presence of digoxigenin-labeled nucleotides to label the amplified product. The PCR product (25 μl) was denatured and allowed to hybridize in solution to a 5′-biotinylated oligonucleotide probe at 37°C in a streptavidin-coated microtiter plate. The bound probe-PCR product was detected by an anti-digoxigenin peroxidase conjugate and by the use of the colorimetric substrate ABTS. In comparison to the β-2 microglobulin standard, we evaluated the presence of amplified product from each PCR reaction.

Statistical analysis

Results from experiments obtained from multiple experiments were reported as the mean ± standard error of the mean (SEM). Significance levels were determined by two-sided Student's *t*-test analysis.

RESULTS

MSCs are a homogenous population of adherent bone marrow cells with a distinct morphology and cell surface protein expression

We sought to establish the morphological differences between MSCs and MDSCs derived from the same bone marrow sample. MDSCs were cultured from the Ficoll fraction of mononuclear cells from marrow in an enriched medium containing 25% serum with 1 μM hydrocortisone. Primary and passage 1 cultures of MDSCs contain a heterogeneous population of hematopoietic and stromal cells (Fig. 1). After 14 days, primary cultures of MDSCs show evidence of hematopoiesis, as indicated by formation of cobblestone areas formation. MSCs were isolated from the Percoll fraction of mononuclear cells and cultured in a medium containing 10% FBS. In contrast to the MDSCs, primary and passage 1 cultures of MSCs appear morphologically as a homogenous population of fibroblastoid cells (Fig. 1). The results demonstrate that the isolation and culture conditions established for MSCs select a distinct population of bone marrow-derived adherent cells.

To compare the expression of cell surface molecules on MSCs and MDSCs obtained from the same donor, we performed flow cytometric analyses. We collected cells from passage 1 cultures and labeled them with monoclonal antibodies, SH-2 and SB-10, that recognize antigens present on the MSCs as well as monoclonal antibodies that recognize antigens present on hematopoietic cells. The identity of SH-2 is being pursued, while SB-10 has been identified as ALCAM (activated leucocyte-cell adhesion molecule) (Bruder et al., in press). The results show that MSCs are a homogenous population of cells that express antigens recognized by SH-2 and SB-10 and the absence of hematopoietic cells. The antigens SH-2 and SB-10 are also present on cells that constitute MDSC cultures (Fig. 2). In contrast to MSCs, the MDSCs were positive for cells expressing both CD14 and CD45 cell surface antigens, demonstrating the presence of hematopoietic cells in these cultures. $CD34^+$ cells were undetected in both passage 1 cultures of MSCs and MDSCs (data not shown). These data emphasize the homogenous characteristic of MSC cultures and indicate the presence of MSC antigens on cells of MDSC cultures.

MSCs show similar cytokine and growth factor expression as MDSCs

We used RT-PCR to compare the steady-state levels of mRNAs for various cytokine and growth factors in the MSCs and MDSCs prepared from the bone marrow cells of the same donor (Fig. 3). We included the amplification of β2-microglobulin mRNA in the analysis in order to monitor equal input of RNA into the reactions. Our initial analysis performed on MSCs included evaluation of mRNA for IL-1 to IL-4, IL-6 to IL-8, and IL-10 to IL-15, LIF, granulocyte colony stimulating factor (G-CSF), GM-CSF, macrophage colony stimulating factor M-CSF, Flt-3 ligand, and stem cell factor (SCF). We were unable to detect mRNAs for IL-2, -3, -4, -10, and -13 in MSCs, although our oligonucleotide primer pairs amplified the correct molecules from appropriate cell lines. For positive PCR signals, we confirmed the amplification products with 5′-biotinylated oligonucleotide primer probes using the PCR-ELISA procedure. The analysis shown in Figure 3 includes only those mRNAs found to be present in MSCs.

We found MSCs maintained in normal culture condition to constitutively express mRNAs for IL-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, LIF, M-CSF, Flt-3 ligand, and SCF (Fig. 3). These results corroborate previous

TABLE 1. Oligonucleotide primers used for the PCR detection of gene transcripts

Oligonucleotide primers/5′-biotinylated probes	Size (bp)	References/accession #
Hu Beta-2 microblobulin	270	Kollman et al. (1994)
5′ TCTGGCCTTGAGGCTATCCAGCGT		
3′ GTGGTTCACACGGCAGGCATACTC		
Probe 5′-biotinylated CATCCATCCGACATTGAAGTTGAC		
Hu IL-1 alpha	808	X02531
5′ ATGGCCAAAGTTCCAGACATGTTTG		
3′ GGTTTTCCAGTATCTGAAAGTCAGT		
Probe 5′-biotinylated TCTGTGTCTCTGAGTATCTCTG		
Hu IL-1 beta	331	M15330
5′ CTTCATCTTTTGAAGAAGAACCTATCTTCTT		
3′ AATTTTGGGATCTACACTCTCCAGCTGTA		
Probe 5′-biotinylated AGTGGTGTTCTCCATGTCCTT		
Hu IL-6	174	Auffray et al. (1994)
5′ GTAGCCGCCCCACACAGACAGCC		
3′ GCCATCTTTGGAAGGTTCAGG		
Probe 5′-biotinylated ATCTCAGCCCTGAGAAAGGAG		
Hu IL-7	681	J04156
5′ ATGTTCCATGTTTCTTTTAGGTATATCT		
3′ TGCATTTCTCAAATGCCCTAATCCG		
Probe 5′-biotinylated AAGAAAACCAGCTGCCCTGGGT		
Hu IL-8	252	M28130
5′ TCTGCAGCTCTGTGTGAAGGT		
3′ TGAATTCTCAGCCCTCTTCAA		
Probe 5′-biotinylated GATTGAGAGTGGACCACACTG		
Hu IL-11	331	M81890
5′ ATGAACTGTGTTTGCCGCCTG		
3′ GAGCTGTAGAGCTCCCAGTGC		
Probe 5′-biotinylated ACGGGGACCACAACCTGGATT		
Hu IL-12 (p40)	378	M65290
5′ TCACAAAGGAGGCGAGGTTC		
3′ TGAACGGCATCCACCATGAC		
Probe 5′-biotinylated GGTGGCTGACGACAATCAGTA		
Hu IL-14	368	L15344
5′ GTGAATGATAAGGCTACTGAGAAG		
3′ GAGCATTTCTCTGTCTGACTTTGAG		
Probe 5′-biotinylated CTGCTCACAAGACAAATGCAGT		
Hu IL-15	356	X94223
5′ CACATTTGAGAAGTATTTCCATCCAGTGC		
3′ GAAGACAAACTGTTGTTTGCTAGGATG		
Probe 5′-biotinylated CAATCTATGCATATTGATGCTAC		
Hu LIF	405	M63420
5′ AACAACCTCATGAACCAGATCAGGAGC		
3′ ATCCTTACCCGAGGTGTCAGGGCCGTAGG		
Probe 5′-biotinylated CAACCTGGACAAGCTATGTGG		
Hu G-CSF	346	E06531
5′ AGCTTCCTGCTCAAGTGCTTAGAG		
3′ TTCTTCCATCTGCTGCCAGATGGT		
Probe 5′-biotinylated CACCTACAAGCTGTGCCACC		
Hu GM-CSF	286	M13207
5′ GTCTCCTGAACCTGAGTAGAGACA		
3′ AAGGGGATGACAAGCAGAAAGTCC		
Probe 5′-biotinylated ATGGCCAGCCACTACAAGCAG		
Hu M-CSF	248	Kollman et al. (1994)
5′ TTGGGAGTGGACACCTGCAGTCT		
3′ CCTTGGTGAAGCAGCTCTTCAGCC		
Probe 5′-biotinylated TAATGGAGGACACCATGCGCT		
Hu Flt-3 ligand	333	U03858
5′ TGCAGCCCAACAACCTATCTC		
3′ GGGCTGAAAGGCACATTTGGT		
Probe 5′-biotinylated TTCAAGATTACCCAGTCACCG		
Hu SCF	177	Auffray et al. (1994)
5′ CTCCTATTTAATCCTCTCGTC		
3′ TACTACCATCTCGCTTATCCA		
Probe 5′-biotinylated TAACCCTCAAATATGTCCCC		

evidence for the presence of many of these cytokines in media conditioned by MSCs (Haynesworth et al., 1996). We found the MDSCs cultured in LTBMC medium to also express these mRNAs (Fig. 3). Under basal conditions, the mRNA for IL-1α and β, G-CSF, and GM-CSF were not detected in cultures of either MSCs or MDSCs. Interestingly, the major amplified product for IL-12 was not detected in MDSCs; however, a minor amplified product of smaller size was observed in both MSCs and MDSCs.

The induction of cytokine expression following exposure to inflammatory molecules such as IL-1 (Aman et al., 1994; Caldwell and Emerson, 1994) is a characteristic response of cultured bone marrow-derived stromal cells. Addition of IL-1α (1, 10, or 100 units/ml) for 4–24 h increased the steady-state level for several cyto-

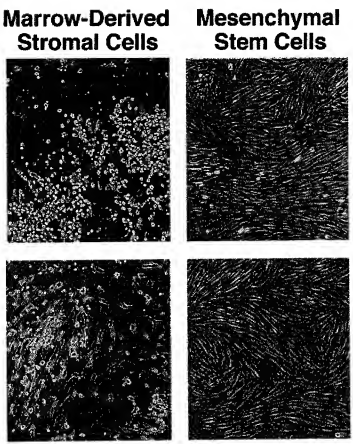

Fig. 1. Morphology of primary (P^0) and passage 1 (P^1) cultures of MDSCs and MSCs. MDSCs were isolated from bone marrow cells by Ficoll fractionation and plated in LTBMC medium. MSCs were isolated from bone marrow cells by Percoll fractionation and plated in MSC medium. P^0 cultures were maintained for 14 days, after which they were trypsinized and replated as P^1 cells. Cultures were maintained as P^1 cells in their respective medium. Photomicrographs are representative of P^0 and P^1 cultures as shown above. ×100.

kine and growth factor mRNAs (data not shown). In Figure 3, we show that a 24 h treatment of MSC cultures with 10 units/ml IL-1α increased the steady-state levels of IL-1α, IL-1β, IL-6, IL-8, IL-11, G-CSF, GM-CSF, and LIF mRNAs. MDSCs cultures treated with IL-1α showed a similar increase in the levels of some of these mRNAs; however, the relative levels of IL-1α, IL-1β, IL-8, IL-11, and LIF mRNA did not appear to change to the same degree in these cultures. IL-1α treatment seemed not to alter the expression of IL-7, IL-12, IL-14, IL-15, M-CSF, Flt-3 ligand, and SCF genes in either MSCs or MDSCs (Fig. 3, lanes 2 and 4). To further confirm the results, we analyzed the expression of IL-1 receptor mRNA on MSCs and on MDSCs and found it to be similar (data not shown), therefore suggesting a comparable effect of IL-1α on both MSCs and MDSCs. Since the composition of the medium used for LTBMC assays is markedly different than MSC media, we examined the effect that switching from MSC to LTBMC medium had on cytokine and growth factor mRNA expression in MSC cultures. Replacement of the MSC media with LTBMC media for 24 h did not alter the steady-state levels of most mRNAs examined with the exception of mRNA for IL-11, which appeared to be lower than those observed in cultures maintained in MSC medium.

We further analyzed the regulation of gene expression of IL-1α, G-CSF, GM-CSF, and LIF by IL-1α. The number of copies of gene transcripts for the individual cytokine and growth factor was determined by analyzing an equal amount of RNA from either untreated MSCs or IL-1α-treated MSCs in RT-PCR reactions, and quantitation of PCR amplified products was performed using 5′-biotinylated oligonucleotide primer probes specific for the genes and PCR-ELISA analysis. The number of gene copies for the cytokines and growth factors were calculated relative to the PCR amplification of known gene copies for the β-2 microglobulin gene. As seen in Table 2, the results not only confirm our observation seen in Figure 3 but also show that treatment of MSCs with IL-1α upregulates the expression of IL-1α, G-CSF, GM-CSF, and LIF by 50-, 800-, 80-, and 350-fold, respectively.

MSCs support hematopoietic differentiation in vitro

The expression of these cytokines and growth factors suggests that MSCs may function in hematopoiesis. Therefore, LTBMCs were established with irradiated MSCs or MDSCs derived from the same marrow sample. Coculture of MDSCs or MSCs with allogeneic immuno-selected $CD34^+$ hematopoietic progenitor cells resulted in the formation of cobblestone areas representative of hematopoietic progenitor cell proliferstion and differentiation (data not shown). Cells derived from LTBMC were plated after 5 weeks in media containing

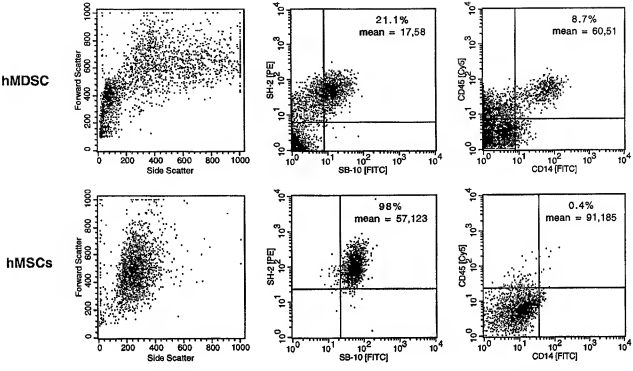

Fig. 2. Flow cytometric analysis of cultured MDSCs and MSCs with monoclonal antibodies. Primary cultures of MDSCs and MSCs were expanded to passage 1 in LTBMC medium and MSC medium, respectively. Populations of these cells were stained with monoclonal antibodies SH-2, SB-10, CD14, and CD45 and analyzed by flow cytometry. The percent (%) and mean fluorescence intensity on the axis were determined by cells in the upper right quadrant. These data are representative of analysis performed on three individual MSC and MDSC cultures.

methylcellulose to assess hematopoietic colony formation. As seen in Figure 4, MSCs from four individual marrow donors maintained long-term culture initiating cells (LTC-IC) in this assay. MSCs from three of four donors were less efficient at maintaining hematopoietic progenitors than the corresponding MDSCs, suggesting some donor variability in this cellular function. This donor variability was particularly evident in the results of assays performed with MSCs from donors 100 and 101. Although these MSCs were cocultured with aliquots of the same $CD34^+$ cell preparation, there was a large difference in the number of hematopoietic colonies that formed in methylcellulose. Experimental controls of LTBMC of $CD34^+$ cells in the absence of adherent monolayer cells did not result in any colony formation. These data demonstrate that cultures of MSCs as a homogenous population have the ability to maintain LTC-IC in hematopoietic long-term culture assay.

DISCUSSION

The bone marrow microenvironment is a complex cellular structure. Investigators have employed primary cells as well as stromal cell lines both from the murine and human bone marrow (Dorshkind and Landreth, 1992; Deryugina and Muller-Sieburg, 1993; Wineman et al., 1993; Roecklein and Torok-Storb, 1995; Mosca et al., 1995) to attain insight towards understanding the cellular and molecular components of the microenvironment and their regulation and maintenance of hematopoiesis. The human stromal cell lines have been established from the LTBMC system (Dexter et al., 1977), while most of the murine cell lines were established by the bone marrow culture systems as described by Dexter et al. (1977) and later modified by Greenberger (1978). These stromal cell lines in general are functionally heterogenous with respect to their ability to sustain B lineage and myeloid cells (Deryugina and Muller-Sieburg, 1993). The murine Whitlock-Witte LTBMC (Whitlock and Witte, 1982) have been crucial for the analysis of the events that regulate proliferation of B lineage cells. Attempts to establish similar human equivalent cell lines have not been successful. A comprehensive analysis (Wineman et al., 1996) designed to assess heterogeneity within the murine stromal compartment was performed which lent experimental support to the hypothesis that the transition from primitive to less primitive stem cells is regulated by distinct stromal cell niches that interact with specific subsets of stem cells. Since similar experiments related to dissecting out the human bone marrow microenvironment are difficult to perform, our understanding has been limited to study of cell lines and to a lesser extent primary cells derived from bone marrow.

Although these studies have increased our knowledge of the bone marrow nonhematopoietic compartment, the precise identity of different cellular compo-

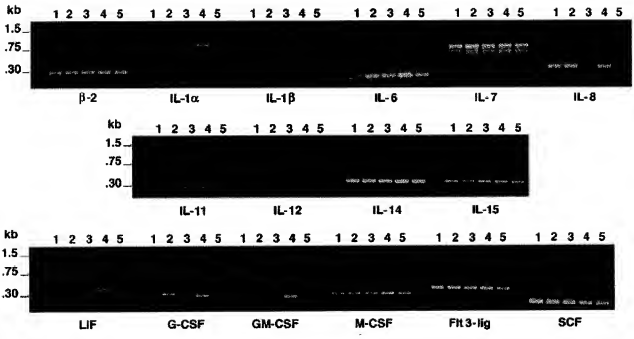

Fig. 3. Gene expression of cytokines and growth factors by MDSCs and MSCs. Total RNA was isolated from MDSCs cultured in LTBMC medium (lanes 1) and LTBMC medium with IL-1α (10 U/ml) for 24 h (lanes 2). Total RNA was also isolated from MSCs cultured in MSC medium (lanes 3), in MSC medium with IL-1α (10 U/ml) for 24 h (lanes 4), and in LTBMC medium for 24 h (lanes 5). Total RNA samples were used for synthesis of cDNA, which was then amplified for 35 cycles using specific primers for cytokines and growth factors genes. Amplified products were analyzed on 2% agarose gels and stained with ethidium bromide. To monitor equal input of RNA in the reverse transcriptase reactions, we used β2-microglobulin gene amplification. Molecular weight markers are represented (kb). These data are representative of results obtained from cultures preapared from at least three individual marrow donors.

TABLE 2. Comparison of regulation of mRNA expression in IL-1-treated MSCs by PCR-ELISA analysis[1]

Cytokines/ growth factors	Transcript copies per nanogram of RNA	
	Untreated	IL-1α-treated
β-2 microglobulin	6,100	6,300
IL-1α	14	7,100
G-CSF	3	2,500
GM-CSF	18	1,500
LIF	8	2,800

[1]Total RNA was prepared from MSCs untreated and treated with IL-1α for 24 h. RT-PCR was performed using total RNA and specific oligonucleotide primers for the cytokines and growth factors. Quantitation of PCR amplified products were performed using 5'-biotinylated oligonucleotide primer probes and the procedure for PCR-ELISA analysis. The number of gene copies for the cytokines and growth factors was calculated relative to the PCR amplification of known gene copies for the β-2 microglobulin gene.

nents and their function in hematopoiesis remain unclear. To this end, we developed methods for the isolation of pluripotent MSCs, an adherent bone marrow cell population that can be expanded in culture without differentiation (Haynesworth et al., 1992b). Here, we examined the morphology, phenotype, and function of MSCs in comparison with MDSCs, the standard cell preparation for in vitro stromal cell culture (Dexter et al., 1977). By the criteria used in this study, we established that culture-expanded MSCs are a homogenous population of fibroblastoid cells distinct from MDSCs which are comprised of both fibroblastic and hematopoietic cells. Like MDSCs, MSCs express numerous cytokines and growth factors and support hematopoiesis in LTBMC, suggesting that MSCs represent an important cellular and functional component of the the stroma.

Our data show that cultures of MSCs are distinct both in morphology and in cellular composition from cultures of MDSCs prepared from the same bone marrow cells. There are several possible explanations for the difference seen in the two cultures, including the methods established for isolation and culture expansion of the cells. First, MSCs are isolated using Percoll (1.073 g/ml) density sedimentation, while MDSCs were cultured following Ficoll-Paque (1.077 g/ml) fractionation. The small difference in the densities between the Percoll and the Ficoll-Paque solutions may be selective for distinct cell populations. The higher density Ficoll-Paque may result in the isolation of cells that sediment through the Percoll solution used for MSC isolation. We believe that the composition of the medium contributes substantially to the disparity of the morphology and composition of the cultures. MDSCs are cultured in LTBMC medium that contains 25% serum (12.5% FBS, 12.5% horse serum) and 1 μM hydrocortisone. In contrast, MSC medium consists of DMEM-LG plus 10% of an FBS selected for the optimal growth of these cells. It is likely that the combination of differences in the

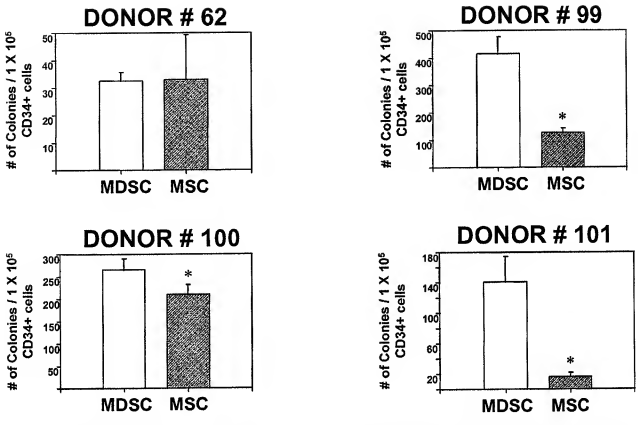

Fig. 4. Comparison of the ability to support hematopoiesis in vitro by MDSCs and MSCs from four donors. Passage 1 cultures of MDSCs and MSCs were irradiated and plated in six-well culture plates. $CD34^+$ cells were isolated from bone marrow and placed in coculture irradiated monolayers of MDSCs and MSCs. Both nonadherent and adherent cells were recovered and cultured in methylcellulose medium for 2 weeks, and hematopoietic colonies greater than 50 cells were counted. The bars represent the mean (±SEM) colony numbers from three individual wells. * Statistical significance ($P < .05$, Student's *t*-test) between MDSCs and MSCs.

isolation method and composition of the culture medium used in the preparation of MSCs and MDSCs contributes to the diverse cellular composition of these cultures.

The hallmark of MDSCs and other stromal cells is their capacity to produce a spectrum of cytokines and growth factors that regulate the proliferation, differentiation, and maintenance of HSCs and its precursors. Stromal cells also increase their synthesis of cytokines such as IL-1α and β, IL-6, G-CSF, and GM-CSF in response to treatment with IL-1α (Paul et al., 1990; Eaves et al., 1991; Sutherland et al., 1991; Cicuttini et al., 1992; Mosca et al., 1995). Haynesworth et al. (1996) previously used ELISA analysis to show that MSCs constitutively secrete IL-6, IL-11, LIF, SCF, and M-CSF and that IL-1α treatment increased the release of IL-6, IL-11, and LIF by MSCs and also induced the secretion of G-CSF and GM-CSF, which was undetectable in untreated cultures. In this study we not only observe the above results but additionally show that MSCs express mRNAs for other cytokines such as IL-7, IL-8, IL-12, IL-14, IL-15, and Flt-3 ligand. Our results also show that MDSCs produce a very similar array of cytokines and growth factors that act on hematopoietic cells. Since our analysis was not quantitative, little can be said about any differences observed in the constitutive levels of these mRNA in MSCs and MDSCs. Interestingly, IL-1α treatment caused an apparent induction of mRNA for IL-1α, IL-1β, and LIF only in cultured MSCs, suggesting distinct differences in the responsiveness of the two cell preparations. The biological relevance of IL-1 in the bone marrow microenvironment is best characterized in models of infection and inflammation. IL-1 mediates, in part, induction of circulating levels of colony stimulating factors, as shown by injecting IL-1 or by blocking IL-1R (Neta et al., 1990). There is also a well-described protective effect of IL-1 in mice after irradiation or cytotoxic drugs (Schwartz et al., 1987). From Figure 3, we observe that MSCs respond to IL-1α distinct from MDSCs especially by autocrine regulation of IL-1. The biological role of IL-1α is primarily as a regulator of intracellular events and a mediator of local inflammation, whereas IL-1β is a systemic, hormone-like extracellular mediator (Dinarello, 1996). Therefore, it is possible that, during inflammation and or infection, MSCs by their cytokines and growth factor expression may be able to influence protective reactions locally as well as at a distance from the bone marrow.

The LTBMC has been established as a standard assay to measure the function of stromal cell cultures in maintaining hematopoiesis in vitro (Dexter et al., 1977; Gartner and Kaplan, 1980). In this study we examined the ability of MSCs to support hematopoiesis in LTBMC and compared the ability of MSCs and MDSCs derived from the same marrow sample to maintain LTC-IC in coculture with $CD34^+$ hematopoietic progenitors. As anticipated, we observed cobblestone areas consisting of hematopoietic cells in cultures of both MSCs and MDSCs, suggesting the development of an in vitro marrow microenvironment. The results of methylcellulose hematopoietic colony assays showed that MSCs are able to maintain and support hematopoietic differentiation of purified $CD34^+$ cells. The results also showed that MSCs were not as efficient as MDSCs in maintaining hematopoiesis in vitro. The number of colonies arising from cocultures of $CD34^+$ cells with MSCs ranged from 11–100% of that for the corresponding MDSC preparation. A possible explanation for the difference in hematopoietic support between the two cultures may lie in the heterogenous nature of the MDSC cultures. It is also possible that other accessory cells like $CD14^+$ hematopoietic cells present in MDSCs may release molecules such as IL-1 and alter the steady-state production of various cytokines by cellular components present in MDSC cultures (Mielcarek et al., 1996). This cell-to-cell interplay may provide an advantage to the MDSC culture in the subsequent support of hematopoietic differentiation. Cultured MSCs should allow us to dissect out the role of such accessory cells starting from selected cell populations. It is also possible that MSCs from different donors are intrinsically different in their synthesis of hematopoietic cytokines, which may dictate the efficiency by which the cells can support hematopoiesis. It is clear from our studies that culture-expanded MSCs maintain LTC-IC in LTBMC and therefore represent an important cellular component of the bone marrow microenvironment.

Caplan (1991) previously postulated that the bone marrow is resident to a population of pluripotent cells capable of differentiating into various mesenchymal lineages. MSCs have been shown to differentiate along osteogenic (Haynesworth et al., 1992a), chondrogenic (Johnstone and Barry, 1996), adipogenic (Pittenger et al., 1996), tendonogenic (Young et al., 1997) lineages to demonstrate their pluripotential characteristic. The results of this study not only provide evidence that MSCs are an important cellular component of the bone marrow microenvironment but also add further to the pluripotent nature of MSCs. Previous work has shown that cultured osteoblastic cells can support hematopoiesis in vitro (Taichman and Emerson, 1994; Taichman et al., 1996), and there is recent data indicating a similar function of cultured adipocytes (Gimble et al., 1996). Therefore, it will be of great interest to investigate the link between lineage differentiation of the MSC and its capacity to maintain hematopoiesis in vitro. The ability to expand MSCs in culture without inducing differentiation makes the MSC an excellent candidate for preclinical and clinical applications in stromal cell therapies.

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METHODS IN MOLECULAR BIOLOGY™

Cardiac Cell and Gene Transfer

Principles, Protocols, and Applications

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Contents

5

Cell Therapy in the Heart

Cell Production, Transplantation, and Applications

Kevin S. Cahill, Catalin Toma, Mark F. Pittenger, Paul D. Kessler, and Barry J. Byrne

1. Introduction

Cardiac dysfunction resulting from various insults to the myocardium can ultimately lead to the development of heart failure. Owing to the limited regenerative capacity of adult cardiac tissue and the lack of a resident cardiac progenitor cell equivalent to the skeletal myoblast, the transplantation of myogenic cells into the myocardium has been investigated as a novel mechanism to repair damaged and dysfunctional myocardium. For maximal impact on cardiac function, the design of cellular therapies must carefully consider the identity of the transplanted cells as well as the method of cell delivery. This review details techniques for the purification of commonly investigated cell grafts, including skeletal myoblasts and mesenchymal stem cells, and the methods to deliver these grafts to the myocardium of laboratory animals.

Since the initial studies documenting the survival of atrial tumor-derived cell grafts in syngeneic myocardium *(1)* a variety of donor cell types have been evaluated (including fetal and adult cardiomyocytes, smooth muscle cells, skeletal muscle myoblasts, and mesenchymal stem cells) in an attempt to determine the most suitable cell type for cardiac repair (for review, *see* **ref. 2**). Skeletal myoblasts are a committed progenitor cell population found in close proximity to the mature skeletal myocytes. This cell population can be easily isolated from an adult muscle biopsy and propagated in tissue culture *(2,15)*. Several reports have documented the ability of skeletal myoblasts to survive in healthy and damaged myocardium and ultimately improve systolic and diastolic function *(3–5)*. Importantly, the ability of myoblasts to be utilized in

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autologous therapies obviates the need for life-long immunosuppression following transplantation.

Pluripotent stem cells capable of differentiation into mature cardiomyocytes have also been proposed for use in cellular therapies *(6)*. Mesenchymal stem cells (MSCs) are an adult bone marrow stem cell population with the potential for multilineage differentiation *(7,8)*. In culture, MSCs undergo rapid proliferation in an undifferentiated state and following exposure to appropriate induction conditions will differentiate into mesodermal lineages, including myocytes and cardiomyocytes *(8–10)*. Since MSCs are easily procured from bone marrow aspirates and readily undergo ex vivo expansion, they also have the potential to be useful in autologous cellular therapies. A functional improvement was demonstrated by Tomita and colleagues *(11)*, who reported that MSC-like cells isolated from rat bone marrow treated with 5-azacytidine could improve heart function in a cyroinjury model of cardiomyopathy.

To have the greatest positive impact on cardiac function, cell delivery strategies must be adapted to the type of myocardial damage that is present. One technique is direct injection of the cellular transplant into a specific region of the myocardium. This technique allows for the delivery of cells to a preselected area and holds promise for cell therapy for localized cardiac injury. For example, the fibrosis resulting from a transmural myocardial infarction can be visualized and then seeded with myogenic cells. This delivery strategy seems most amenable to situations in which a severe obstruction in arterial blood flow would preclude vascular delivery.

Alternatively, cell grafts can be delivered to the myocardium via the vasculature. It has been demonstrated that cell transplants will migrate across the capillary wall to engraft in the myocardium when arterially delivered *(12–14)*. In large animals and humans, this can be accomplished through the use of a catheter-mediated intracoronary infusion, whereas in rodents other techniques are needed to overcome the exceedingly small size of the vasculature. One technique for arterial cell delivery in rodents is an intraventricular cell injection. Of the cells delivered to the left ventricular cavity, a small percentage will enter the coronary circulation. Importantly, arterial delivery techniques allow the cells to engraft in all areas of the myocardium receiving coronary artery perfusion. This may allow for a maximal therapeutic effect when the myocardium is globally damaged, as is seen in muscular dystrophies with cardiac involvement or end-stage heart failure with global cardiomyopathy.

2. Materials

2.1. Myoblast Isolation and Culture Materials

1. 1X Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, with 4.5 g/L glucose, without sodium pyruvate (Cellgro from Mediatech, Herndon, VA).
2. Phosphate-buffered saline (PBS), 1X solution without calcium and magnesium (Cellgro).

3. Fetal bovine serum (FBS) and horse serum (HS). Inactivate complement by incubation at 56°C for 30 min (Cellgro).
4. Penicillin and streptomycin stock solution (Cellgro), containing 10,000 IU per 100 mL penicillin and 10,000 µg/mL streptomycin.
5. Chick embryo extract (CEE, Gibco-BRL), for use in the myoblast culture media.
6. Myoblast culture media: DMEM with 20% FBS, 0.5% CEE, 5 mL of the penicillin/streptomycin stock solution. Sterilize the media by passage through a 0.22-µm filter.
7. Collagenase and trypsin-EDTA: trypsin-EDTA (Cellgro) 10X stock solution and 1% collagenase (Sigma St. Louis, MO).
8. Nylon mesh, 50-µm sterile.
9. Collagen-coated 60-mm tissue culture dishes.

2.2. Mesenchymal Stem Cell Isolation and Culture Materials

1. 1X DMEM with L-glutamine, with 1.0 g/L glucose, without sodium pyruvate (Cellgro).
2. FBS selected for MSC outgrowth (BioWhittaker, Walkersville, MD).
3. 100X MEM nonessential amino acid stock solution (Cellgro).
4. Mesenchymal stem cell culture media: DMEM (low glucose) with 10% selected FBS, 5 mL of the penicillin/streptomycin stock solution, and 5 mL of the nonessential amino acid solution. Sterilize the media by passage through a 0.22-µm filter.
5. 25-Gauge syringe, 3-mL needle, sterile nylon mesh.

2.3. Myoblast Delivery Materials

1. Standard surgical instruments and materials.
2. 29-Gauge insulin syringe.
3. Rodent ventilator (Harvard Apparatus, Holliston, MA).

3. Methods

3.1. Isolation of Myoblasts from Skeletal Muscle

3.1.1. Myoblast Harvest

The following procedure should be performed in a certified tissue culture hood, and all instruments and solutions should be properly sterilized.

1. Starting with 200–400 mg of freshly excised skeletal muscle, use scissors to cut the muscle into square pieces approx 2 mm in length.
2. Add the pieces to a 15-mL sterile conical tube containing 10 mL of PBS with 1X trypsin-EDTA and 1% collagenase. Incubate in a 37°C water bath for 15–30 min and gently vortex the tube once during the incubation. Stop the enzymatic digestion by transferring the supernatant into a 50-mL sterile conical tube containing 10 mL of DMEM supplemented with 10% FBS.
3. Triturate the neutralized solution by pipeting up and down several times with a 25-mL pipet. This should disperse the larger material present in the solution. Any

additional aggregates can be removed by passing the solution through a 30–60-µm nylon mesh.
4. Collect the cells by centrifugation at 200*g* for 5 min. Resuspend the cells in 1 mL of myoblast growth media and count the cells using a hemocytometer. Plate the cells at an approximate density of 1×10^5 cells/60-mm collagen-coated dish.
5. Culture cells in a 37°C incubator with 5% CO_2. One day after plating, perform two successive media changes to remove any nonadherent cell debris (*see* **Notes 1** and **2**).

3.1.2. Growth of Myoblast Cultures

The myoblasts will proliferate and become confluent in approx 2–3 d. Do not allow the cells to reach confluence, as they will begin to differentiate. Myoblasts should be trypsinized and replated at 1:4 dilutions when they become approx 80% confluent. Media should be changed every 2 d if the cells are not being split.

3.2. Isolation of Mesenchymal Stem Cells from Bone Marrow

The selection of MSCs from bone marrow is based on the phenomenon that MSCs readily adhere to tissue culture dishes, whereas hematopoietic and other contaminating cell types do not attach and/or do not readily expand in selected lots of serum. The purity and growth characteristics of MSC cultures may be variable and species-dependent. All MSC cultures should be characterized by flow cytometry and differentiation assays *(8)*. We have had the best results with rat and human MSC cultures, although certain strains of murine cultures are also useful.

3.2.1. MSC Harvest

This protocol will detail the harvest of MSCs from mice and should be performed in a tissue culture hood to minimize contamination of cultures. Isolation of MSCs from other species can be accomplished using this protocol with minor modifications.

1. Obtain 10 freshly dissected femurs. Dissect any remaining musculature from the femurs using forceps and scissors. Use an alcohol swab to clean the femurs and remove any remaining ligaments.
2. Using either scissors or a small bone cutter, carefully remove the proximal- and distalmost portions of the femur to provide access to the medullary cavity within the shaft.
3. Flush the bone marrow from the medullary cavity with PBS using a 3-mL syringe with a 25-gauge needle. This is accomplished by inserting the needle in the shaft and then rotating and moving the needle vertically while dispensing the PBS to completely dislodge the bone marrow from the medullary cavity. Collect the flow

through in a 15-mL conical tube.
4. Centrifuge the aspirate at 900*g* for 5 min and resuspend the cell pellet in 2-mL of MSC culture media. Pass the cellular solution through a 25-gauge needle several times to disrupt any cellular clumps and then pass the solution through nylon mesh to remove any contaminating bone fragments.
5. Plate the cells at an appropriate density so that after several days of growth MSC colonies are evenly spaced. This will vary with the strain of mouse used and the effectiveness of the harvest.
6. Change the medium daily for the first week to remove contaminating nonadherent cell types (*see* **Note 3**).

3.2.2. MSC Culture

1. Continue media changes every 2–3 d to ensure removal of hematopoietic cells. Colonies of MSCs should be seen 5–10 d after plating.
2. The rate of MSC proliferation will vary with the strain of mouse used. The MSCs should be trypsinized and replated at 1:4 dilutions when they become approx 80% confluent.

3.3. Delivery of Cellular Graft to Murine Myocardium

The following surgical techniques assume that the researcher has been trained in the basics of microsurgery in rodents. All surgeries are performed with the assistance of a dissecting microscope. Prior to in vivo myocardial delivery, the cells should be marked to allow for later identification in the myocardium. This can be accomplished through the use of membrane or DNA dyes such as CM-DiI or DAPI or through replication-defective recombinant viruses carrying marker genes. Our laboratory routinely utilizes recombinant adeno-associated virus vectors for the long-term transduction of MSCs and myoblasts.

3.3.1. Preparation of Cellular Grafts

1. Obtain the appropriate number of MSC- or myoblast-containing culture dishes. Remove the culture media and wash the cells twice with PBS prewarmed to 37°C.
2. Add 2–3 mL of PBS containing 1X trypsin-EDTA. Incubate at 37°C for 2–5 min. Monitor the reaction by microscopic examination of the culture for dissociated cells. Dislodge the cells by gently tapping the flask as necessary. MSC cultures may require an increased incubation time to remove all the cells completely. When all of the cells are in suspension, stop the enzymatic digestion by addition of 3 mL of DMEM with 10% FBS. Transfer the suspension to a 15-mL conical tube.
3. Pellet the cells by centrifugation at 1000*g* for 5 min. Resuspend the pellet in 1 mL of PBS and count the cells using a hematocytometer.
4. Again, pellet the cells by centrifugation at 1000*g* for 5 min. Resuspend the cell pellet in the appropriate volume of PBS to give a final concentration of 10^6 cells

per 25–50 µL. The cells should be injected as soon as possible following resuspension. Maintain the cells on ice until injection.

3.3.2. Direct Myocardial Injection of Cellular Grafts

The murine myocardial wall is exceeding thin, and care must be taken to ensure that the material is not injected into the ventricular cavity. The following protocol is designed to be performed in a mouse; however, most cardiac manipulations are technically easier to perform in a rat, and this protocol can easily be adapted to larger rodents. Sterile instruments and aseptic technique should be used at all times to decrease the chance of infection.

1. Appropriately anesthetize the animal. We routinely use ketamine and xylazine, although various other anesthetics are effective.
2. Using clippers first and then a razor, remove the hair from the thorax and the left axilla. Clean the shaved area with a 0.5% betadine solution and alcohol.
3. Place the animal on a positive pressure ventilator. This can be done by the passage of an 18-gauge angiocatheter through the oral cavity into the trachea (*see* **Note 4**). Connect the angiocatheter to the ventilator tubing. The ventilator settings depend on the weight of the animal. We routinely set the respiration rate to 115 breaths per minute and adjust the tidal volume to approximate normal respiratory activity visually. Secure the animal in the supine position with the upper limbs extended away from the thorax to a temperature-controlled operating surface. Monitor body temperature through the use of a rectal thermometer.
4. A lateral thoracotomy is performed to expose the heart. Make a 1–2-cm coronal incision through the skin approx 1 cm inferior to the left axilla. Use a fine-point high temperature cautery to ligate the superficial vessel running from the axilla inferiorly. Next use scissors to make an incision between two successive ribs. The intercostal musculature is only several millimeters thick, and care must be taken to ensure that the lung is not damaged. Cauterize any perforated vessels as needed. The thoracic wall can now be retracted as necessary to expose the myocardium adequately.
5. Once the area of interest in the heart is localized, use a 29-gauge needle to inject the cell suspension into the wall of the myocardium. Avoid advancing the needle into the ventricular cavity.
6. To close the incision, use 6-0 prolene to approximate the ribs and musculature. Care must be taken to ensure that the lungs are not inadvertently damaged and that the thoracic wall is secure and air-tight. Prior to completion of the closure, aspirate any air or fluid from the thorax by a 22-gauge angiocatheter connected to light suction. Finally, close the skin with 5-0 suture.
7. The animal can be weaned from the ventilator as the anesthetic begins to wear off. This process can be accelerated by the administration of a respiratory stimulant. Once the animal is spontaneously breathing, remove the tracheal tube.
8. The animal should be placed in a temperature-controlled recovery room and given antibiotics and pain medication following surgery.

3.3.3. Arterial Delivery of Myoblasts

Access to the left ventricular cavity can be obtained by a transdiaphragmatic injection. The murine heart is easily visible through the semitransparent diaphragm following a midline abdominal incision and retraction of the liver. Alternatively, several techniques can be utilized to increase coronary cell delivery involving dissection and occlusion of the great vessels. These techniques, however, require a highly trained microvascular surgeon and are beyond the scope of this review. The following procedure is designed for use in mice.

1. Anesthetize the animal by inhalation of isoflurane until the animal is unconscious. Monitor the breathing rate of the animal. Slow, labored breathing may indicate that too much anesthetic has been given.
2. Using clippers and then a razor, remove the hair from the abdomen. Clean the abdomen with a 0.5% betadine solution.
3. Use scissors to make a midline incision through the abdominal skin extending from the xyphoid process to about 2 cm inferiorly. Next, make an incision in the abdominal wall musculature along the linea alba.
4. Use a retractor to reflect the abdominal walls laterally. Locate the liver. Depress the liver with a cotton swap and use scissors to cut the falciform ligament. The diaphragm and inferior surface of the heart should be visible.
5. Using an insulin syringe containing the cells to be injected, advance the needle through the diaphragm and myocardial wall while pulling back gently on the plunger of the syringe. When the needle has entered the high-pressure left ventricle, a rush of bright red blood will enter the syringe. Retract the needle slightly and then inject the contents (*see* **Note 5**).
6. Following injection, remove the needle from the heart and apply immediate pressure to the injection site using a cotton swap. This step is essential to decrease the chance of pneumothorax. The injection site in the myocardial wall will seal spontaneously.
7. Close the abdominal wall incision with 6-0 prolene suture and then the skin with 5-0 suture.
8. The animal should be placed in a temperature-controlled recovery room and given antibiotics and pain medication following surgery.

4. Notes

1. The yield of myoblasts decreases with the age of the animal. Additionally, various techniques that injure the skeletal muscle 2–3 d before harvest can result in an increase in the yield of myoblasts.
2. Myoblast enrichment of the primary cultures can be accomplished based on the observation that myoblasts take longer to adhere to tissue culture dishes than fibroblasts. Thus, removing and replating the nonadherent myoblast suspension after 30–60 min of the initial plating will enrich for myoblasts. An increase in the cell yield can also be obtained by redigesting the muscle reminants following the initial enzymatic digestion of the muscle pieces.

3. The bone marrow aspirate can be centrifuged through a density gradient to remove contaminating cell types prior to plating. We centrifuge the aspirate at 1100*g* for 30 min in a 1.073 g/mL Percoll gradient. The nucleated cells collected at the interface and the upperlayer are then washed and plated.
4. Intubation can be accomplished in a mouse through the oral cavity or via a tracheotomy. To locate the laryngeal inlet, place the mouse supine on a platform inverted at approx 45 degrees. While using forceps to open the oral cavity, shine a bright light in the area of the mouse's neck. Look into the mouth and you will see a small circular light in the oral cavity. This is the laryngeal inlet and can be used as a guide to ensure that the esophagus is not inadvertently entered.
5. The maximum number of cells that can be delivered in a single intraventricular injection is approx 10^6. We routinely delivery this cell number in a volume of 100 µL. An increase in cell number may result in lethal cellular emboli. However, successive injections can be performed to increase the total number of cells that reach the myocardium. Prior to injection, it is useful to filter the cells through a 30-µm nylon mesh to avoid injection of large cell clumps that may produce lethal emboli. During injection it is helpful to have an assistant raise the xyphoid process with forceps and depress the liver with a cotton swab to increase visibility of the heart.

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6

Cardiac Cell Transplantation

Protocols and Applications

Steven M. White and William C. Claycomb

1. Introduction

Cellular transplantation into the heart is an emerging field with numerous applications for designing new therapeutic strategies for treating various types of heart disease. The two primary applications of cellular transplantation are to generate new functional myocardium and to deliver therapeutic agents such as growth factors into diseased hearts. Cardiac cellular transplantation experiments have been performed using different cell types, including cardiomyocyte cell lines *(1)*, primary cardiomyocytes *(2,3)*, skeletal myocytes *(4–6)*, and fibroblasts *(7)*. In performing cardiac cellular transplantation experiments, various animal models have been used to assess the efficacy of autologous *(8)*, syngeneic *(9)*, allogeneic, and xenogeneic *(1,10)* cellular transplants. Many investigators are currently focusing on using embryonic stem (ES) cells *(10,11)* and bone marrow-derived stem cells *(12,13)* for in vitro differentiation into cardiomyocytes for transplantation, or as direct sources for cardiac cellular transplantation. In order for cells to be useful for transplantation into injured myocardium, they must be able to survive in the recipient tissue, have limited capacity for replication, and become physically and electrically coupled to each other and the neighboring host myocardium so that they may all contract synchronously.

We have used the adult swine as a large animal model for studying the effects of cellular transplantation into injured myocardium *(1)*. Because the heart size and coronary anatomy closely resemble those of humans, the porcine animal model can provide valuable information regarding the efficacy of cellular

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transplantation as a therapy for heart disease. During the course of the experiments the animals undergo several procedures, depending on whether the cells are being transplanted into normal or infarcted myocardium. Cardiac catheterization is used to introduce an embolization coil into the left anterior descending (LAD) branch of the left coronary artery to induce a nonlethal myocardial infarction. The subsequent epicardial cellular injections are made using a median sternotomy to access the heart. There are several major issues related to performing cellular transplantation experiments including proper personnel and facilities, selection of cells to use for transplantation, and care for the animals used in the experiments. This chapter provides a detailed protocol for cardiac cellular transplantation in the porcine model and describes applications for this new and exciting therapy.

2. Materials

2.1. Personnel

For further information, *see* **Note 1**.

1. Cardiothoracic surgeon.
2. Cardiologist.
3. Anesthetist.
4. Cell culture technician (*see* **Note 2**).
5. Animal care providers (*see* **Note 3**).

2.2. Facilities

For further information, (*see* **Note 4**).

1. Animal care facility for large animals (adult swine).
2. Cardiac catheterization suite.
3. Surgical suite with anesthesia equipment.
4. Postoperative monitoring facility.

2.3. Cells

The following cell types have been used for transplantation into the heart:

1. Freshly isolated cardiomyocytes (fetal, neonatal, adult) ***(1,7,14)***.
2. Cells from established cardiomyocyte cell lines (HL-1, AT-1) ***(1)***.
3. Skeletal myocytes ***(4–6)***.
4. ES cells (mouse) ***(10,11)***.
5. Bone marrow-derived stem cells ***(12,13)***.

2.4. Drugs, Media, and Reagents

2.4.1. Cell Transport for Transplantation Experiments

1. Cell culture medium specific for the cell type being used.
2. Ice for transporting cells.

2.4.2. Induction and Maintenance of General Anesthesia

1. Ketamine.
2. Acetylpromazine.
3. Pentobarbital.
4. Isoflurane.
5. Lidocaine.
6. Diltiazem.
7. Isotonic (normal) saline solution for intravenous administration.

2.4.3. Cardiac Catheterization

1. All drugs and solutions listed under **Subheading 2.4.2.**
2. Labetalol.
3. Lidocaine (*see* **Note 5**).
4. Diltiazem infusion bag (*see* **Note 6**).
5. Epinephrine.
6. Surgical soap
7. Iodine solution (Betadine).
8. Sterile water.
9. Renografin contrast solution.

2.4.4. Median Sternotomy Procedure

1. All drugs and solutions listed under **Subheading 2.4.2.**
2. Surgical soap.
3. Iodine solution (Betadine).
4. Sterile water.

2.4.5. Cellular Transplantation

1. Joklik's medium.
2. Ice.
3. Warm isotonic saline solution.

2.4.6. Postoperative Period

1. All drugs and solutions listed in **Subheadings 2.4.2.** and **2.4.3.** should be available.

2.4.7. Immunosuppression

1. FK-506.
2. Cyclosporine.
3. Prednisone.

2.4.8. Organ Harvesting

1. All drugs and solutions listed in **Subheadings 2.4.2.** and **2.4.3.**
2. Heparin.
3. Prednisone.

2.5. Equipment

2.5.1. Cell Transport for Transplantation

1. Sterile vial(s) (*see* **Note 7**).
2. Container for ice.

2.5.2. Induction and Maintenance of General Anesthesia

1. Sterile syringes (10 mL).
2. Sterile 22-gauge needles.
3. Sterile 18 gauge iv catheters.
4. Sterile iv connector tubing
5. Laryngoscope and blades.
6. Sterile endotracheal tubes (different sizes).
7. Pressure-controlled ventilator and anesthesia machine.
8. Arterial line kit.
9. Monitor capable of showing electrocardiogram (ECG), mean arterial blood pressure (MABP), and arterial O_2 saturation.
10. ECG electrodes.
11. Pulse oximetry electrode.

2.5.3. Cardiac Catheterization

1. All equipment listed in **Subheading 2.5.2.**
2. Functional cardiac catheterization suite with fluoroscopy.
3. Sterile drapes and towels.
4. Sterile 4 × 4-cm gauze sponges.
5. Sterile towel clips.
6. Sterile scalpel and scalpel blades.
7. Cordis introducer sheath (5–6-F; Cordis Co.).
8. Catheterization kit.
9. 5-F H1 embolization catheter.
10. Cooper embolization coil (0.5 × 10 mm; Cook Co.).
11. Guidewire.
12. Defibrillator (DC).

2.5.4. Median Sternotomy Procedure

1. All equipment listed in **Subheading 2.5.2.**
2. Sterile drapes and towels.
3. Sterile 4 × 4-cm gauze sponges.
4. Sterile towel clips.
5. Suction apparatus with sterile tips and tubing.
6. Sternal saw.
7. Sterile surgical instruments including the following:
   a. Scalpel blades.
   b. Scalpel handle.
   c. Traumatic and atraumatic forceps of different sizes.

d. Curved and straight Metzenbaum scissors.
e. Suture scissors.
f. Needle holders of different sizes.
g. Hand-held retractors.
h. Sternal retractor.

8. Electrocautery apparatus
9. Sutures including the following types:
   a. 2-0 silk.
   b. 2-0 silk "pop-offs."
   c. 2-0 silk ties.
   d. 7-0 proline sutures.

2.5.5. Cellular Transplantation

1. Doppler thickening probes.
2. All surgical instruments listed in **Subheading 2.5.4., #7.**
3. All sutures listed in **Subheading 2.5.4., #9.**
4. Sterile 1-mL tuberculin syringes.
5. Sternal wires.
6. Sterile dressings and tape.

2.5.6. Postoperative Period

1. All equipment listed in **Subheading 2.5.2.** for anesthesia and monitoring.

2.5.7. Organ Harvesting

1. All equipment listed in **Subheading 2.5.2.** for anesthesia and monitoring.
2. All surgical equipment listed in **Subheading 2.5.4.**

3. Methods

3.1. Cell Transport for Transplantation

1. Prior to the transplantation experiments, the cells should be maintained according to the protocol for the specific cell type.
2. For transporting cells to the operating room for transplantation, cells should be suspended in sterile cell culture medium in a sterile vial and transported on ice (*see* **Note 8**).

3.2. Induction and Maintenance of General Anesthesia

1. Determine the weight (in kg) of the animal to be used (*see* **Note 9**).
2. Induce general anesthesia by administering 7 mg/kg ketamine im and 0.2 mg/kg acetylpromazine im followed by 25 mg/kg pentobarbital im.
3. Position the animal in the supine position on the procedure table and secure all limbs to the table.
4. Intubate the animal by using a laryngoscope to pass an endotracheal tube through the vocal cords into the main bronchus (*see* **Note 10**).
5. Connect the endotracheal tube to the pressure-controlled ventilator.

6. Ventilate the animal with 1–2% isoflurane.
7. Verify that the animal is under anesthesia and unresponsive to painful stimuli.
8. Obtain peripheral intravenous access (in any limb) by inserting a subcutaneous infusion catheter connected to intravenous tubing and a bag of normal saline solution.
9. Insert a peripheral arterial line for monitoring arterial blood pressure and blood gases during the cellular transplantation procedure.
10. Begin constant infusions of 3 mg/kg lidocaine iv and 2.5 mg/kg diltiazem iv to control ventricular rate and the onset of atrial arrhythmias.
11. Place ECG electrodes so that a 3-lead ECG may be used to monitor cardiac electrical activity during the procedure.

3.3. Cardiac Catheterization to Induce a Myocardial Infarction

1. Two days prior to the creation of the myocardial infarction, give the animals 200 mg labetalol (a β-adrenergic antagonist) each day in their feed to reduce the chance of mortality during the catheterization procedure.
2. Prepare each animal for the catheterization by following the protocol outlined in **Subheading 3.2.** *(1–11)* except for the placement of an arterial line (#9).
3. Give an iv infusion of normal saline supplemented with lidocaine (3 mg/kg over 20 min) and diltiazem (120 mg/h) during the procedure and for at least 2 h after the procedure to prevent ventricular arrhythmias (*see* **Note 11**).
4. Spread apart the rear legs and secure to the table.
5. Prepare the right groin for a surgical incision using sterile technique.
   a. Scrub the groin with surgical soap and sterile water using sterile gauze sponges.
   b. After scrubbing the area for at least 5 min, wipe the area clean with sterile towels to remove the soap lather.
   c. Apply iodine solution (Betadine) to the incision site using sterile gauze in a circular fashion starting from the site of the incision and spreading out to the periphery of the groin.
6. Locate the intended incision site by palpating the right femoral artery.
7. Create a sterile field around the intended incision site in the right groin by placing sterile towels and sheets around the incision site.
8. All subsequent steps should be performed in a sterile manner.
9. Make an approx 3-mm incision over the right common femoral artery using a sterile scalpel blade and create a small tunnel in the subcutaneous tissue with a hemostat.
10. Using the Seldinger technique, cannulate the femoral artery, insert the guidewire through the cannula, and advance the catheter over the guidewire.
11. Pass the catheter through the introducer sheath and advance the tip of the catheter to the aortic root under direct fluoroscopic guidance.
12. Perform coronary angiography by injecting Renografin contrast solution through the catheter so that the coronary anatomy may be studied (*see* **Note 12**).

13. Depending on the individual animal's coronary anatomy, either the terminal portion of the LAD or one of the obtuse marginal branches of the left circumflex artery may be chosen for the creation of the myocardial infarction (*see* **Note 13**).
14. Advance the 5-F H1 embolization catheter through the catheter already in place in the aortic root and maneuver it into the left main coronary artery and subsequently into either the middle to distal LAD artery or the proximal portion of a large obtuse marginal branch of the left circumflex artery.
15. With the embolization catheter in place and confirmed using angiography, deploy a Cooper embolization coil (0.5 × 10 mm) (Cook Co.) using a 0.035-in. guidewire.
16. ECG confirmation of myocardial infarction is demonstrated immediately by S-T segment elevation.
17. Symptomatic cardiac arrhythmias resulting from the deployment of the embolization coil should be treated according to the rhythm, and efforts should be made to resuscitate animals developing lethal arrhythmias using the following (*see* **Note 14**):
    a. DC cardioversion.
    b. 1 mg/kg epinephrine.
    c. 3 mg/kg lidocaine.
18. Perform repeat angiography to confirm that the vessel containing the embolization coil is occluded.
19. Remove the catheter apparatus from the animal.
20. Maintain pressure on the femoral artery for at least 10 min after the catheter is removed.
21. Monitor the animal and allow it to recover by following the protocol in **Subheading 3.6.**
22. Allow the animal to recover for 1 mo following the myocardial infarction, and prior to the cellular transplantation procedure.

3.4. Procedure for Performing a Median Sternotomy

1. Pepare each animal for the median sternotomy by following the protocol outlined in **Subheading 3.2.** *(1–11)*.
2. Begin preparing the chest for the incision by shaving any hair from the sternal and surrounding areas (*see* **Note 15**).
3. Using sterile water, surgical soap, and sterile gauze sponges, scrub the entire chest for at least 5 min (*see* **Note 16**).
4. Use sterile towels to dry the chest, removing all soap and water.
5. Apply iodine solution (Betadine) using sterile gauze sponges (*see* **Note 17**).
6. Create a sterile field by applying sterile towels and drapes around the intended incision site (*see* **Note 18**).
7. Perform all subsequent procedures using sterile technique by personnel properly scrubbed and dressed according to sterile technique (*see* **Note 19**).
8. Ensure the animal is under general anesthesia by applying a painful stimulus.
9. Ensure the sternal saw is working prior to making the incision.

10. Using a sterile scalpel blade, make a midline incision over the entire sternum.
11. Dissect down to the sternum using electrocautery to maintain hemostasis.
12. Ensure the entire sternum is accessible for splitting with the sternal saw.
13. Stop ventilation so that the lungs are deflated just long enough for the sternum to be split (*see* **Note 20**).
14. Position the sternal saw at the superior border of the sternum in the sternal notch and proceed inferiorly, splitting the sternum.
15. Continue ventilations once the sternum is completely split.
16. Insert a sternal retractor between the edges of the divided sternum and slowly open to provide an adequate working space.
17. After visualizing the heart, lift the anterior pericardium using atraumatic forceps with the help of the assistant, and cut longitudinally using Metzenbaum scissors.
18. Once an adequate view of the heart is obtained through the opening in the pericardium, suture the cut edges of the pericardium and tie to the sternal spreader using "pop-off" silk sutures.
19. Approximately three sutures on each edge of the cut pericardium are adequate to create a pericardial well in which the remainder of the procedure will take place.

3.5. Cellular Transplantation into the Myocardium

1. Transport the cells to be used for transplantation to the operating room on ice in small sterile vials at a concentration of 10^6 cells/20 µL culture medium (*see* **Note 21**).
2. The myocardial injection sites should be decided in advance depending on whether the heart is normal or if a myocardial infarction has been created (*see* **Note 22**).
3. For hearts with myocardial infarctions, the infarct zone is identified visually and confirmed using Doppler thickening probes placed on the surface of the myocardium (*see* **Note 23**).
4. Suture two probes (one over both normal and infarcted myocardium) to the epicardial surface with 7-0 proline sutures. These probes will be left in place for the duration of the animal's recovery until it is sacrificed 4–6 wk following the procedure.
5. For each injection, draw 100 µL of cells into a 1-mL tuberculin syringe with a 26-gauge needle.
6. Inject cells through the epicardium into the myocardium at various locations (*see* **Note 24**).
7. After each injection, place a 7-0 proline suture to mark each injection site for future gross and histologic examination.
8. Give each heart at least one sham injection of 100 µL culture medium without cells to serve as a control.
9. After the injections are made, fill the pericardium with warm saline solution and observe the heart for any bleeding.
10. Remove the saline with suction, and remove the sutures holdi g the edges of the cut pericardium to the sternal retractor.
11. Leave the pericardium open (*see* **Note 25**).

12. Place drains within the pericardium and exit through the inferior portion of the incision.
13. Pass sternal wires (6) on needles through each half of the sternum at the same position on each side to accommodate approximation of the cut sternum.
14. Once all the sternal wires are in position, close the chest by grasping all the sternal wires and pulling the sternal borders together.
15. With the assistant holding the chest closed with the sternal wires, the surgeon takes each wire separately and twists it on itself so that it will hold its position and not slip.
16. After all the wires are twisted, cut each wire with surgical wire cutters, leaving an approx 0.5-cm tail of twisted wire.
17. Using a needle driver, fold the wire tails down toward the sternum so that they will not injure the overlying tissue.
18. After the ribcage is closed, place subcutaneous proline sutures followed by superficial sutures.
19. Dress the wounds by placing sterile gauze over the entire incision and securing the dressing with tape.

3.6. Postoperative Care of the Animal

1. Once the cellular transplantation procedure is complete, allow the animal to recover for 4 h under general anesthesia with the arterial line in place to monitor blood pressure.
2. During this recovery period, titrate bretylium and diltiazem to relative bradycardia or the presence of significant ventricular arrhythmias (*see* **Note 26**).
3. After 4 h, discontinue the anesthesia and allow the animal to regain consciousness.
4. Once the gag reflex begins to return, extubate the animal.
5. Leave ECG leads in place for a few hours to monitor cardiac electrical activity.
6. Return the animals to the animal care facility and maintain with an appropriate diet according to the institution's guidelines.
7. Allow animals to recover for 1 mo prior to harvesting the heart (*see* **Note 27**).

3.7. Immunosuppression Therapy

1. Immediately following the cellular transplantation procedure, start the animals on immunosuppressive therapy consisting of the following:
   a. 0.2 mg/kg FK-506 by oral gavage.
   b. 15 mg/kg cyclosporine by oral gavage.
   c. 0.35 mg/kg prednisone by oral gavage.
2. Maintain the animals on this immunosuppressive therapy from the time of cellular transplantation until they are sacrificed for harvesting of the heart (*see* **Note 28**).

3.8. Harvesting the Heart for Analysis

1. Prepare each animal for the median sternotomy to remove the heart by following the protocol outlined in **Subheading 3.2.** ***(1–11)*** with the exception of #9.
2. Open the chest through the same incision used for the previous sternotomy. Cut the wires holding the sternum together with wire-cutters, allowing the sternum to open.

3. Insert the sternal retractor to open the chest, exposing the heart in the cut pericardial sac.
4. Cut the heart free of any adhesions that may have developed since the last procedure.
5. Give the animal 1000 U/kg heparin iv to prevent blood clotting in the microvasculature of the heart.
6. Excise the heart by cutting the proximal aorta, pulmonary arterial trunk, and pulmonary veins, and allow the animal to exsanguinate.
7. The heart tissue is now ready to be processed according to the types of analyses desired (*see* **Note 29**).

3.9. Future Applications

Cardiac cellular transplantation is an exciting new therapy for the treatment of various types of heart disease. There have been many studies examining the efficacy of cellular transplantation as a means of generating functional myocardial tissue following an infarction. Although the data concerning the degree of functional improvement in ventricular function are not clear, it is clear that cellular transplantation into injured myocardium will play a major role as a therapeutic strategy in the near future. There are still many issues to be investigated with regard to the types of cells to be used for transplantation and the optimal method of delivery. Although this chapter has focused on the use of epicardial injections for cellular transplantation, other methods such as endocardial injections and intraarterial injections *(15)* have also been investigated. Instead of using direct injection as the method of delivery, it could be possible to deliver cells with altered cell surface protein expression into the systemic circulation and have them migrate to the heart. This could potentially make cellular transplantation minimally invasive.

An exciting area of cellular transplantation is the use of stem cells derived from various sources. The plasticity of stem cells makes them promising prospects for cardiac cellular transplantation with regard to their wide differentiation potential and ease of genetic manipulation. One application for genetically altering cells for transplantation is manipulating cells in vitro to reduce or alleviate their immunogenicity. Currently, there is controversy regarding appropriate sources of stem cells to be used in cellular transplantation. By genetically altering stem cells from different sources to render them nonimmunogenic, graft rejection could be alleviated and the donor pool for cells would be greatly increased, allowing for allogeneic and even xenogeneic transplantation.

Another potential application for cardiac cellular transplantation is in the treatment of certain congenital heart diseases. Most of the current studies in cardiac cellular transplantation focus on regenerating damaged myocardium as a result of an infarction. Although this is a promising use of cellular transplantation, the use of cellular therapy as a treatment for congenital heart malforma-

tions is also a potential therapeutic option. With the continuing development of early *in utero* diagnosis of cardiac malformations and growing experience with fetal surgery, it is possible that using cellular transplantation to reconstruct malformations or to deliver therapeutic agents that will alter cardiac morphogenesis directly to the heart will be a feasible therapy in the near future.

Cellular transplantation in the heart is a promising therapy not only for generating functional myocardial tissue but also for delivering growth factors and other therapeutic agents. It will be possible to alter cells genetically so that they constitutively or inducibly secrete therapeutic agents into the local tissue for purposes such as inducing angiogenesis, altering myocardial remodeling, or inhibiting local inflammation. These types of cellular therapies could play significant roles in many cardiovascular diseases, both acquired and congenital.

4. Notes

1. The personnel listed represent specialists in each area and are suggested, not required. For instance, the cardiologist or surgeon may be proficient in anesthesia, so that a separate person to deliver anesthesia may not be required. It is important that all of the personnel involved be experienced in dealing with the particular animal being used in the experiments.
2. The cell culture technician is the person responsible for providing the cells at the time of the transplantation experiments.
3. The type of animal care providers required will vary depending on the individual institution. We recommend that a licensed veterinarian be used as a consultant in the care of the animals, although veterinary technicians will be providing the daily care.
4. Although different institutions will vary in the facilities available for these types of experiments, all the facilities listed in **Subheading 2.2.** should be equipped similarly to those used for humans with respect to anesthesia and monitoring equipment.
5. Lidocaine (3 mg/kg) is given over 20 min.
6. 500 mg diltiazem (120 mg/h) is made in small infusion bags by the hospital pharmacy.
7. These can be any type of sterile vial (1–2 mL) routinely used in tissue culture.
8. For our experiments, we used cells suspended at a concentration of 10^6 cells/20 µL culture medium. Different numbers and concentrations of cells may be used depending on the goal of the experiment.
9. Animal weights are usually expressed in kilograms to facilitate drug dosing.
10. The person performing the intubation should be familiar with the unique upper airway anatomy of the adult swine.
11. Although the combination of lidocaine and diltiazem should be sufficient to control postoperative arrhythmias, additional agents such as β-receptor antagonists may also be useful.
12. Make sure enough images are obtained to ensure a complete and detailed view of the entire coronary anatomy.

13. Choosing a coronary branch that is too large could result in a lethal infarction. Also, if the goal of the experiment is to produce an animal with heart failure, choosing too small a vessel may not result in sufficient myocardial impairment to progress into clinical heart failure.
14. Prolonged resuscitation of animals with lethal arrhythmias is not advised.
15. Blot the chest with tape to remove any excess hair.
16. The animal should be scrubbed from the neck and shoulders down to the abdomen, and the entire chest should be cleaned from the most lateral regions (midaxillary lines in humans).
17. Apply the iodine solution by "painting" the chest, starting with the intended incision site and moving to the periphery of the region to be sterile. Take care not to reapply iodine solution to an area already covered.
18. Four sterile towels are typically placed first around the four sides of the incision site, creating a rectangle with the intended incision site in the middle. Adjacent towels are connected using sterile towel clips. Next, various large drapes are applied to cover exposed portions of the animal, table, and any neighboring equipment that could serve as a potential source of contamination.
19. Surgical personnel wearing caps and masks should scrub (at least 5 min) and wear sterile gowns and gloves as if this were a human surgical procedure.
20. This requires coordination between the surgeon and the person in charge of ventilations (and anesthesia).
21. The volume of cells in culture medium taken to the operating room depends on the number of injections intended for each cell type.
22. In experiments using hearts with infarcted myocardium, it is often desirable to make injections of cells into normal myocardium, in the middle of the infarct zone, and on the periphery of the infarct zone, close to normal myocardium.
23. Probes over normal myocardium should demonstrate ventricular thickening during systole (corresponding to the QRS complex on the ECG), whereas the probes over the infarcted myocardium will show thinning of the wall during ventricular systole owing to the presence of scar tissue. These probes measure ventricular wall thickness over time. The output of data from the ECG, Doppler thickening probes, and MABP can be measured simultaneously and viewed on the same video monitor.
24. When making injections, if localized cellular transplants are desired, it is important to pull back on the plunger of the syringe prior to injecting the cells to ensure that the tip of the needle is not in a vessel or the ventricular cavity. This is especially important when injecting cells into infarcted myocardium because of its thinner wall. If blood is seen when retracting the plunger, reposition the tip of the needle and reconfirm that the tip of the needle is in the myocardial interstitium.
25. Leaving the pericardium open allows the Doppler thickening probes and drains to exit through the incision site and remain attached to the animal postoperatively.
26. This requires close monitoring of the ECG to look for symptomatic arrhythmias.
27. This period is at least 4 wk to allow myocardial scar formation.
28. In this adult swine model, approx 60–70% of the animals survive the myocardial infarction and continue to recover for approx 1 mo.

29. Depending on the type of analyses desired, portions of the heart may be immediately processed for RNA isolation or fixed for histology or immunohistochemistry, depending on the particular experiment.

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RAPID COMMUNICATION
Biomaterials & Bioengineering

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Tissue-engineered Neogenesis of Human-shaped Mandibular Condyle from Rat Mesenchymal Stem Cells

ABSTRACT

The temporomandibular joint is susceptible to diseases and trauma that may ultimately lead to structural degeneration. Current approaches for replacing degenerated mandibular condyles suffer from deficiencies such as donor site morbidity, immunorejection, implant wear and tear, and pathogen transmission. The hypothesis of this study was that a human-shaped mandibular condyle can be tissue-engineered from rat mesenchymal stem cells (MSCs) encapsulated in a biocompatible polymer. Rat bone marrow MSCs were isolated and induced to differentiate into chondrogenic and osteogenic cells *in vitro*, and encapsulated in poly(ethylene glycol)-based hydrogel in two stratified layers molded into the shape of a cadaver human mandibular condyle. Eight weeks following *in vivo* implantation of the bilayered osteochondral constructs in the dorsum of immunodeficient mice, mandibular condyles formed *de novo*. Microscopic evaluation of the tissue-engineered mandibular condyle demonstrated two stratified layers of histogenesis of cartilaginous and osseous phenotypes. The current approach is being refined for ultimate therapeutic applications.

KEY WORDS: TMJ, osteochondral, tissue engineering, cartilage, bone.

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INTRODUCTION

Temporomandibular disorders (TMD) affect approximately 30 million individuals in the United States alone, with more than one million new patients added each year (LeResche, 1997; Stohler, 1999). TMDs may manifest as pain, myalgia, headaches, and structural destruction known as degenerative joint disease (Okeson, 1996). The temporomandibular joint (TMJ), like other synovial joints, is also prone to rheumatoid arthritis, injuries, and congenital anomalies (LeResche, 1997; Stohler, 1999). The severe form of TMJ-associated degenerative disorders necessitates surgical replacement of the mandibular condyle (Sarnat and Laskin, 1991). Currently available materials for surgical replacement of the mandibular condyle—such as autologous, allogenous, xenogenous grafts or artificial prosthesis—suffer from deficiencies such as donor site morbidity, limited tissue supply, immunorejection, potential transmission of pathogens, and complications of wear and tear (Henning *et al.*, 1992; Wolford and Karras, 1997; Baird and Rea, 1998; Bell *et al.*, 2002). A tissue-engineered mandibular condyle from the patient's own tissue-forming cells should overcome these deficiencies.

Previous attempts to tissue-engineer mandibular condyles have utilized several meritorious approaches (for review, see Glowacki, 2001) that inspired various components of the present work. For instance, chondrocytes or osteoblasts encapsulated in various hydrogels survive *in vitro* fabrication and synthesize cell-associated extracellular matrices (Poshusta and Anseth, 2001; Schliephake *et al.*, 2001; Springer *et al.*, 2001; Weng *et al.*, 2001). Increasingly sophisticated scaffold design influences cell differentiation patterns (Hollister *et al.*, 2002; Sherwood *et al.*, 2002). The premolded shape of the mandibular condyle is retained after marrow-derived osteoblasts are seeded in scaffolds consisting of poly-lactic-glycolic acid or natural coral (Weng *et al.*, 2001; Chen *et al.*, 2002; Abukawa *et al.*, 2003). However, an unmet challenge is to tissue-engineer a mandibular condyle from adult stem cells that differentiate into both chondrogenic and osteogenic lineages, an approach that not only mimics the developmental processes of the mandibular condyle, but also is necessary for ultimate clinical applications. Stem cells are necessary because full-thickness osteochondral defects, such as those in severe arthritis, heal poorly in the absence of corresponding tissue-forming cells (Hunziker, 2002; Lietman *et al.*, 2002). Adult mesenchymal stem cells have advantages over embryonic stem cells for tissue engineering of the mandibular condyle, because adult mesenchymal stem cells (MSCs) can be obtained from the same individual and readily induced to differentiate into both chondrogenic and osteogenic cells (Caplan, 1994).

Hydrogels are hydrophilic polymers capable of absorbing biological fluids while serving as a three-dimensional scaffold, thus providing tissue-forming cells with a mimicked environment of the extracellular matrix (Lee and Mooney, 2001). Polyethylene-glycol-based hydrogel, such as that used

Figure 1. Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells. (A) Recovery process of a tissue-engineered mandibular condyle after eight-week *in vivo* implantation in immunodeficient mouse. (B,C) Harvested osteochondral construct retained the shape and size of the cadaver human mandibular condyle mold. (D) Acrylic model of a cadaver human mandibular condyle. (E) Palyurethane mold used to load the cell/palymer suspensions.

in the present work, is biocompatible and has been shown to maintain the viability of encapsulated cells (Poshusta and Anseth, 2001; Burdick *et al.*, 2002). The objective of the present study was to tissue-engineer a human-shaped mandibular condyle from a single population of rat mesenchymal stem cells that had been induced to differentiate into chondrogenic and osteogenic lineages.

MATERIALS & METHODS

Harvest and Culture of MSCs

Rat bone-marrow MSCs were harvested from two- to four-month-old (200-250 g) male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA). Following the rats' death by CO_2 asphyxiation, the tibia and femur were dissected, and whole bone-marrow plugs were flushed by means of an 18-gauge needle and 10-mL syringe loaded with Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Biocell, Rancho Dominguez, CA, USA) and 1% antibiotic-antimycotic (Gibco, Carlsbad, CA, USA). Marrow samples were mechanically disrupted by passage through 16-, 18-, and 20-gauge needles. Marrow cells were centrifuged, re-suspended in serum-supplemented medium, counted, plated at 5 x 10^7 cells/100-mm culture dish, and incubated in 95% air/5% CO_2 at 37°C for 2 wks, with fresh medium change every 3-4 days. Upon reaching 80-90% confluence, primary MSCs were trypsinized, counted, and passaged at a density 5-7 x 10^5 cells/100-mm culture plate. The animal protocol was approved by the institutional Animal Care Committee.

Treatment of MSCs with Chondrogenic and Osteogenic Differentiation Factors

The same population of first-passage MSCs was treated separately with chondrogenic or osteogenic specially formulated medium. The chondrogenic medium was supplemented with 10 ng/mL TGF-β1, whereas the osteogenic medium contained 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate. Cultures were incubated for 1 wk in 95% air/5% CO_2 at 37°C, with fresh medium change every 3-4 days.

Hydrogel Preparation and Cell Photoencapsulation

Poly(ethylene glycol) diacrylate (PEGDA) (Shearwater, Huntsville, AL, USA) was dissolved in PBS supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco) to a final solution of 10% w/v. A biocompatible ultraviolet photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (Ciba, Tarrytown, NY, USA) was added to the PEGDA solution to make a final concentration of 0.05% w/v. After trypsinization and counting, MSC-derived chondrogenic and osteogenic cells were re-suspended separately in the polymer/photoinitiator solution at a concentration of 5 x 10^6 cells/mL.

For *in vivo* experiments, a 200-μL aliquot of cell/polymer suspension containing MSC-derived chondrogenic cells was loaded in the human mandibular condyle-shaped polyurethane mold (ps. 1D, 1E), followed by photopolymerization with UV light at 365 nm (Glowmark, Upper Saddle River, NJ, USA) for 5 min (Elisseeff *et al.*, 2000). MSC-derived osteogenic cells suspended in polymer/photoinitiator solution were loaded to occupy the remainder of the mold (approx. 400 μL), followed by photopolymerization. For the *in vitro* assay, a 100-μL aliquot of cell/polymer suspension containing either MSC-derived chondrogenic cells or MSC-derived osteogenic cells was loaded in tissue culture inserts (diameter, 5 mm), followed by photopolymerization.

In vivo Implantation and *in vitro* Incubation of Hydrogel Constructs

Following photopolymerization, the osteochondral construct was removed from the mold and washed with PBS supplemented with 1% antibiotics. After anesthesia of severe combined immunodeficient (SCID) mice (four- to five-week-old males) (Harlan) by I.P. injection of 100 mg/kg ketamine plus 5 mg/kg xylazine, the osteochondral constructs were implanted into dorsal subcutaneous pockets formed by blunt dissection. Four fabricated constructs were implanted into 2 SCID mice. Three experimental constructs contained MSC-derived chondrogenic and osteogenic cells encapsulated in 2 stratified layers of poly(ethylene glycol) diacrylate hydrogel, whereas the fourth construct, containing untreated MSCs, served as a control.

To demonstrate chondrogenesis and osteogenesis *in vitro*, we removed the resulting constructs (6 samples *per* group) from the tissue culture inserts and incubated them in six-well tissue culture plates with either chondrogenic or osteogenic medium, respectively. Control samples consisted of 6 constructs encapsulating untreated MSCs and 6 constructs with no cells. Control constructs were incubated with DMEM/FBS without exposure to chondrogenic or osteogenic factors. MSC monolayer cultures (6 culture plates *per* group) were incubated with chondrogenic or osteogenic medium, or with DMEM/FBS as control. All hydrogel constructs and monolayer cultures for the *in vitro* assay were incubated statically at 95% air/5% CO_2 at 37°C for 4 wks, with fresh medium change every 3-4 days.

Harvest of Tissue-engineered Mandibular Condyles and Histologic Phenotyping

Eight wks following subcutaneous implantation, tissue-engineered osteochondral constructs were harvested from SCID mice. Following the animals' CO_2 asphyxiation, an incision was made in the dorsum of the SCID mice (Fig. 1A). After careful separation

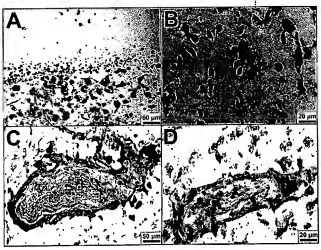

Figure 2. Photomicrographs of histologic phenotypes of a representative tissue-engineered mandibular condyle following 8 wks of *in vivo* implantation. (A) Von Kossa silver-stained section showing the interface between chondral and osseous layers. Multiple mineralization nodules were present in the osseous layer (lower half of the photomicrograph), but absent in the chondral layer (upper half of the photomicrograph). (B) Positive safranin O staining of the chandrogenic layer was represented by intense red, indicating the synthesis of negatively charged cartilage-specific glycosaminoglycans in the extracellular matrix. (C) H&E-stained section of the osteogenic layer showing a representative island structure consisting of MSC-differentiated osteoblast-like cells on the surface and in the center. (D) Positive toluidine blue staining of an island structure in the osseous layer.

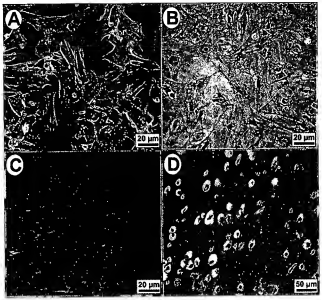

Figure 3. Chondrogenesis driven by MSC-derived chondrogenic cells in *ex vivo* samples. (A) Positive reaction of MSC-derived chondrogenic cells to safranin O in monolayer culture following four-week treatment with chondrogenic medium containing TGF-β1. (B) Monolayer culture of MSCs from the same population as in (A), cultured for 4 wks with DMEM/FBS but without TGF-β1, showed no positive reaction to safranin O. (C) Positive reaction of PEG hydrogel encapsulating MSC-derived chondrogenic cells to safranin O, demonstrating the presence of cartilage-specific glycosaminoglycans after four-week incubation in chondrogenic medium containing TGF-β1. (D) PEG hydrogel encapsulating the same population of MSCs as in (C), but without exposure to TGF-β1, showed negative reaction to safranin O.

from the surrounding fibrous capsule, the tissue-engineered mandibular condyles were removed (Figs. 1B, 1C), rinsed with PBS, fixed in 10% formalin overnight, embedded in paraffin, and sectioned in the sagittal plane and parallel to the long axis of the construct at 5-μm thickness according to standard histological procedures. Sequential sections were stained with hematoxylin and eosin, toluidine blue, von Kossa's silver stain, and safranin O/fast green so that osseous and cartilaginous phenotypes could be distinguished. The same histologic preparations were used for *in vitro* constructs. Monolayer cultures were stained with either safranin O or von Kosssa and alkaline phosphatase stain. A fresh mixture of Naphthol, DMF (N, N-Dimethylformamide), Tris-HCl, and red violet LB salt (Sigma) stained monolayer cultures for alkaline phosphatase, followed by standard von Kossa staining.

RESULTS

Tissue-engineered mandibular condyles formed *de novo* in the dorsum of immunodeficient mice (Fig. 1A) from osteochondral constructs consisting of a single population of MSC-derived chondrogenic and osteogenic cells encapsulated in two stratified layers of PEG-based hydrogel. The tissue-engineered mandibular condyle measured 11 x 4 x 7 mm (length and height measurements in Figs. 1B and 1C, respectively), virtually the same size as the polyurethane mold of the human cadaver mandibular condyle (*cf.* Fig. 1E). Gross examination and physical manipulation indicated that the tissue-engineered mandibular condyles were opaque, firm, and retained the macroscopic shape of the cadaver human mandibular condyle from which the polyurethane mold was made (Figs. 1D, 1E).

Nodules of mineral deposits in the osteogenic layer containing MSC-derived osteogenic cells encapsulated in PEG-based hydrogel were revealed with von Kossa silver staining (the lower half of Fig. 2A). In contrast, the chondrogenic layer, consisting of MSC-derived chondrogenic cells encapsulated in PEG-based hydrogel, lacked mineralization nodules (the upper half of Fig. 2A). The chondrogenic layer contained sparse chondrocyte-like cells within abundant extracellular matrix that reacted positively to safranin O (Fig. 2B). Multiple islands of dark-stained structures with H & E were present in the osteogenic layer, consisting of MSC-derived osteogenic cells encapsulated in PEG-based hydrogel (Fig. 2C). Osteoblast-like cells resided on the surface and in the center of these islands. These island structures reacted positively to toluidine blue (Fig. 2D). The control construct, consisting of hydrogel encapsulating untreated-MSCs, reacted negatively to safranin O, von Kossa, and toludine blue staining (data not shown).

Marrow-derived MSCs treated with chondrogenic medium in monolayer culture exhibited positive reaction to safranin O after four-week incubation in chondrogenic medium (Fig. 3A), whereas the same population of MSCs cultured without TGF-β1 showed negative reaction to safranin O (Fig. 3B). Positive reaction to safranin O was also evident after encapsulation of MSC-derived chondrogenic cells in PEG-based hydrogel, especially in the matrix after four-week incubation of the

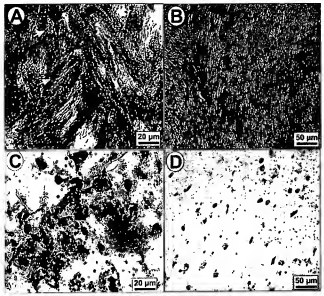

Figure 4. Osteogenesis driven by MSC-derived chondrogenic cells in ex *vivo* samples. (A) Positive reactions of MSC-derived osteogenic cells to alkaline phosphatase (white arrow) and von Kossa silver stain (green arrow) following four-week incubation in osteogenic medium. (B) Monolayer culture of MSCs from the same population as in (A), cultured for 4 wks with DMEM/FBS but without osteoinduction factors, showed no positive reaction to either alkaline phosphatase or von Kossa silver stains. (C) Von Kossa silver-stained section of PEG-hydrogel encapsulating MSC-derived osteogenic cells showing mineral nodules. (D) The same population of MSCs encapsulated in the PEG-hydrogel construct without exposure to osteogenic medium showed no evidence of mineralization by von Kossa silver staining.

hydrogel-MSC construct in the chondrogenic medium (Fig. 3C). In contrast, the same population of MSCs without exposure to chondrogenic medium prior to encapsulation in the hydrogel showed negative reaction to safranin O (Fig. 3D).

Marrow-derived MSCs in monolayer culture incubated for 4 wks in osteogenic medium reacted positively to both alkaline phosphatase (white arrow in Fig. 4A) and von Kossa silver stain (green arrow in Fig. 4A), indicating their osteogenic phenotype and mineral deposition, respectively. In contrast, the same population of MSCs without exposure to osteogenic medium showed negative reaction for both alkaline phosphatase and von Kossa staining (Fig. 4B). Further, the osteogenic constructs, consisting of PEG-hydrogel encapsulating MSC-derived osteogenic cells incubated in osteogenic medium for 4 wks, showed the formation of mineral nodules and positive reaction to von Kossa silver stain (Fig. 4C), whereas the same population of MSCs without exposure to osteogenic medium showed negative reaction to von Kossa staining (Fig. 4D). The PEG hydrogel encapsulating MSC-derived chondrogenic cells showed negative reaction to osteogenic markers such as von Kossa stain, whereas PEG hydrogel encapsulating MSC-derived osteogenic cells demonstrated negative reaction to chondrogenic markers such as safranin O (data not shown). In addition, control hydrogel constructs without any cells reacted negatively to both chondrogenic and osteogenic markers (data not shown).

DISCUSSION

The present approach to the tissue-engineering of a human-shaped mandibular condyle with stratified chondrogenic and osteogenic layers from a single population of rat bone marrow mesenchymal stem cells addresses several issues in this field. The outcome of tissue-engineered mandibular condyles from MSC-derived chondrogenic and osteogenic cells represents another step toward therapeutic applications of total joint replacement in comparison with approaches using isolated mature chondrocytes or osteoblasts (Niederauer *et al.*, 2000; Weng *et al.*, 2001). The differentiation of MSCs into chondrogenic cells and osteogenic cells *in vitro* is consistent with previous work (*e.g.*, Goldberg and Caplan, 1994; Schaefer *et al.*, 2000; Gao *et al.*, 2001), leading to active chondral and osseous matrix syntheses. Although the present encapsulation density of both MSC-derived chondrogenic and osteogenic cells at 5 x 10^6/mL has led to *in vivo* chondrogenesis and osteogenesis, the optimal densities of both MSC-derived chondrogenic and osteogenic cells should be determined.

Analysis of the present data demonstrates that MSC-derived chondrogenic and osteogenic cells continued their phenotypic differentiations both *in vitro* and *in vivo*. This is remarkable, since MSC-derived chondrogenic and osteogenic cells were encapsulated into the shape of a human mandibular condyle with a dimension of 11 x 4 x 7 mm. The *in vitro* osteogenic potential of MSC-derived osteogenic cells in the present work is evidenced by their positive reactions to alkaline phosphatase and von Kossa staining. *In vitro* chondrogenesis in the present work is evidenced by positive reaction to safranin O, a cationic dye that binds to cartilage-specific glycosaminoglycans such as chondroitin sulfate and keratan sulfate (Lammi and Tammi, 1988; Mao *et al.*, 1998; Wang and Mao, 2002). On the other hand, chondrogenesis and osteogenesis *in vivo* were demonstrated by strong safranin O labeling of the chondrocytes' extracellular matrix, and positive reaction to von Kossa staining as well as by the formation of dark HE-stained island structures occupied by osteoblast-like cells, respectively. Matrix synthesis by MSC-derived chondrogenic and osteogenic cells in 2 stratified, and yet integrated, layers of PEG hydrogel corroborates previous findings from the use of similar PEG-based hydrogel systems (Elisseeff *et al.*, 2000; Poshusta and Anseth, 2001; Burdick and Anseth, 2002; Halstenberg *et al.*, 2002; Martens *et al.*, 2003). In the present study, histological examination of the chondrogenic layer revealed abundant safranin-O-positive matrices of chondrocyte-like cells. In contrast, the present observation of osteoblast-like cells on both the surface and the center of toluidine-blue-positive island structures warrants further characterization for genetic and biochemical osteogenic markers. The selection of eight-week *in vivo* implantation was based on both our preliminary data and the anticipated clinical requirement for the shortest possible *ex vivo* incubation time (Temenoff and Mikos, 2000; Gao *et al.*, 2001; Altman *et al.*, 2002).

The use of a uniform polymer such as PEG-based hydrogel for both chondral and osseous components of osteochondral constructs has additional advantages, such as the ease of fabrication, and improved adhesion and interpenetration between the 2 layers (Lu and Anseth, 1999; Lee and Mooney, 2001; Nguyen and West, 2002). In the present study, physical manipulation of the *ex vivo* photopolymerized constructs and

the harvested *in vivo* constructs failed to separate the 2 layers. PEGDA is biocompatible, biodegradable, and FDA-approved for several medical applications (Fu *et al.*, 2002). Despite a somewhat slow degradation rate, degradation of PEGDA in the present study is evident from both cell-associated matrix synthesis and formation of distinctive microscopic structures in both the chondrogenic and osteogenic layers. A common tendency associated with seeding cells in prefabricated three-dimensional scaffolds is their localization in the scaffold's surface (*e.g.*, Abukawa *et al.*, 2003). In the present study, loading MSC-derived chondrogenic and osteogenic cells in PEG hydrogel solution before photopolymerization likely has allowed for relatively homogenous cell distribution. On the other hand, copolymer may be necessary to promote differential needs of chondrogenesis and osteogenesis (Schaefer *et al.*, 2000; Gao *et al.*, 2001; Sherwood *et al.*, 2002).

Much additional work is needed before tissue-engineered mandibular condyles are ready for therapeutic use in patients suffering from osteoarthritis, rheumatoid arthritis, injuries, and congenital anomalies. A meritorious approach is *in vivo* growth factor delivery to maintain phenotypic differentiations of chondrogenic and osteogenic cells (Martin *et al.*, 1999; Blunk *et al.*, 2002; Burdick *et al.*, 2002; Pei *et al.*, 2002a). The mechanical strength of tissue-engineered mandibular condyles must be enhanced so that they are capable of withstanding the mechanical stresses that normal condyles experience. Mechanical stresses with tailored peak magnitudes and frequencies are capable of modulating bone and cartilage growth at different levels of organization in both the appendicular and craniofacial skeletal lineages (Carter *et al.*, 1998; Goldstein, 2002; Mao, 2002; Wang and Mao, 2002; Kopher and Mao, 2003). Recently, both hydrodynamic stresses and bioreactors have been shown to enhance the biophysical properties of tissue-engineered cartilage constructs (Buschmann *et al.*, 1995; Vunjak-Novakovic *et al.*, 1999; Mauck *et al.*, 2000; Altman *et al.*, 2002; Davisson *et al.*, 2002; Pei *et al.*, 2002b). The enhancement of mechanical properties of tissue-engineered mandibular condyles likely will be a critical step toward clinical applications. Nonetheless, the present findings represent a proof of concept for further development of tissue-engineered mandibular condyles.

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